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Phytochemical analysis of *Ranunculus* species with possible involvement in Equine Grass Sickness

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List of abbreviations

A	*Ranunculus acris*
B	*Ranunculus bulbosus*
C	Control samples
$CDCl_3$	Deuterated chloroform
D_2O	Deuterium oxide
E	Equine grass sickness samples
EGS	Equine grass sickness
G	Golder Manor Farm samples
iNOS	Inducible nitric oxide synthase
MeOD	Deuterated Methanol
NMR	Nuclear magnetic resonance
PCA	Principal component analysis
PC	Principal component
PLS	Partial least squares
PLS-DA	Partial least squares – discriminant analysis
R	*Ranunculus repens*
TMS	Tetramethylsilane
TSP	Trimethylsilyl propionate

1. Introduction

1.1. Equine Grass Sickness

Equine grass sickness (EGS), or equine dysautonomia, is a polyneuropathy affecting the central, peripheral and enteric nervous systems of grazing horses.[1]
Although EGS has been recognized for nearly 100 years the cause has not been definitively determined.[2] Recent studies showed that intoxication with *Clostridium botulinum* type C is involved in EGS[3], but it is very likely that EGS has a multifactorial aetiology.

1.1.1. Symptoms

There are two distinct clinical presentations of EGS. The first form, acute grass sickness, presents as colic, with rapid development of clinical signs. This form is invariably fatal and all cases die or require euthanasia, usually within 7 days. Symptoms of acute grass sickness are dysphagia, hyper salivation (see Fig. 1), complete anorexia, nasogastric reflux and absence of gut sounds. Other clinical signs not attributed to the gastrointestinal tract are depression, dehydration, tachycardia and muscle tremors.[4]

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[1] McGorum et al. (2000)
[2] Wylie et al. (2009)
[3] Cottrell et al (1999)
[4] Wylie et al. (2009)

Fig. 1: A horse showing marked hypersalivation (common cases of acute grass sickness).[5]

The second clinical presentation of EGS is chronic grass sickness, which is mainly characterized by weight loss or dysphagia. Other clinical signs associated with failure of normal gastrointestinal function are mild colic, inappetence and severe weight loss (development of a “wasp-waist”, see Fig. 2). Systemic clinical signs not attributable to gastrointestinal tract dysfunction are sweating, depression, rhinitis sicca and behavioral changes like “playing with drinking water”. Survival of this form may be possible. It is unusual for EGS to recur in recovered individuals, although there are sporadic unconfirmed reports.[6]

[5] Wylie et al. (2009)

[6] Wylie et al. (2009)

Fig. 2: The typical clinical presentation of a chronic grass sickness case displaying marked weight loss and the appearance of a ‘‘wasp-waist.’’[7]

1.1.2. Geographic distribution

EGS occurs most frequently in Great Britain, where the first outbreak was recognized in eastern Scotland in 1909. Epidemiologic studies have suggested there are identifiable high-risk areas throughout the United Kingdom with a high proportion of cases occurring in Scotland, but with cases also occurring in England and Wales. EGS is considered to occur rarely in Ireland, with only three reported cases. EGS has also been reported in many countries in Western Europe, including France, Germany, Hungary, Norway, Sweden, Austria, Switzerland, the Netherlands, Belgium, Denmark, Finland, Cyprus and Luxembourg.

The prevalence is considered to be highest in countries geographically close to Great Britain, in particular Germany and Belgium.[8]

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[7] Wylie et al. (2009)

1.1.3. Risk factors

The currently favored theory is that EGS is a form of toxico-infectious botulism, resulting from *Clostridium botulinum* producing toxins locally within the horse's intestinal tract.[9] According to Hunter et al., *C. botulinum* Type C was shown present in 48% of ileum samples from horses with EGS compared to 7% with control samples from healthy horses. Since *C. botulinum* type C toxin in gastrointestinal contents can also be found in with healthy horses[10] it is likely that other factors are involved in the aetiology of EGS.

The following clinical signs are common to both botulism and EGS: anorexia, colic, dysphagia, hypersalivation, tachycardia and weight loss. Mydriasis, profound myasthenia and respiratory distress are features unique to botulism.

As the name suggests, there is a strong association between the development of EGS and access to grazing. There are only rare isolated reports of cases in horses without access to fresh grass.[11] Although cases may occur in any month, peaks in the diagnoses occur during the spring and early summer.[12] Young horses and those who have recently moved premises are at significantly increased risk.[13] Animals in contact with previous cases are at a 10-fold lower risk.[14]

Results of epidemiological studies also showed that premises on loam and sand soils had increased rates of recurrence, whereas premises on chalk and other soil types

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[8] Wylie et al. (2009)

[9] Newton et al. (2004)

[10] Hunter et al. (1999)

[11] Wylie et al. (2009)

[12] Newton et al. (2004)

[13] McCarthy et al. (2001)

[14] Newton et al. (2004)

had significantly reduced rates of recurrence. Soil inhabitants such as earthworms and moles, are able to burrow more freely though sand and loam soil types, causing disruption and increasing the rate of soil contamination. Therefore it is more likely for *C. botulinum* to reside in these soil types and hence bringing the bacterium into contact with grazing horses more frequently.

1.1.4. EGS and *Ranunculus*

The aim of this research was to find out if *Ranunculus* samples from EGS sites are significantly different compared to *Ranunculus* samples from sites, where EGS has not occurred. Our hypothesis is that *Ranunculus* might be another factor involved in the aetiology of equine grass sickness. The following facts support this hypothesis:

- Fresh *Ranunculus* contains Ranunculin, a glycoside that is enzymatically hydrolyzed to produce Protoanemonin, a toxic metabolite that can cause blistering and gastrointestinal irritation. Therefore buttercups might be able to evoke lesions in the gastrointestinal tracts of the horses. This may allow the bacterium *Clostridium botulinum* to enter the bloodstream.
- EGS occurs only in horses with access to fresh grass. *Ranunculus* contains the irritant metabolite Protoanemonin that dimerizes into the non-toxic Anemonin when the plant is dried. Therefore intoxication is only possible if horses ingest fresh *Ranunculus*.
- EGS occurs more often in young horses. In general, grazing animals reject *Ranunculus* because of its acrid taste, but when other food is scarce or when young animals are first turned out to pasture they tend to overeat them. Under these conditions some animals may develop a taste for the plants and

continue to eat them, even deliberately choosing them when other food is available.[15]

- Ingestion of *Ranunculus* by horses can cause excitation, muscle tremor, paralysis, conjunctivitis, ablepsia, deafness and colic.[16] Some of these symptoms are also typical for EGS.
- The peak of EGS outbreak is in spring. During this time Protoanemonin levels in the plants are usually elevated.

In order to test this hypothesis *Ranunculus* samples were collected at 12 sites where EGS has occurred as well as 9 control sites where EGS has not occurred. Another aim of these studies was to find out if there are seasonal changes in the metabolite profiles. Therefore 10 samples have been collected at one site, but at different times of the year.

1.2. *Ranunculus* **- species**

1.2.1. *Ranunculus repens*

R. repens is also called creeping buttercup. The plant reaches heights from 10 to 50 cm and has a creeping rhizome. The leaves are three-lobed and they grow out of the nodes. The sepals are close to the petals and the pedicles are sulcate.[17]

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[15] Cooper, Johnson (1988)

[16] Habermehl, Ziemer (1999)

[17] Lauber et al. (2007)

1.2.2. *Ranunculus acris*

Other common names for *R. acris* are common buttercup; field buttercup, tall buttercup and crowfoot. *R. acris* is a 30 to 80 cm high, perennial herb with a short rhizome.[18]The stems are erect or may creep along the ground but do not form roots. The basal leaves have long stalks and the upper ones short stalks. The leaf blades are hairy and deeply divided into 2-7 lobes which are deeply indented and toothed.[19]

1.2.3. *Ranunculus bulbosus*

R. bulbosus is also called St. Anthony's turnip. It is a perennial, hairy plant that grows from a rounded or flattened stem tuber which bears fleshy roots. The stems are usually erect up to 40 cm high and bear deeply cut, three-lobed leaves of which the central lobe is long-stalked.[20] Unlike *R. acris* and *R. repens*, *R. bulbosus* has a bulb-like swollen corm. *R. bulbosus* has been used as a homoeopathic drug. Its indications go from menstrual pain to coronary diseases and rheumatism.[21]

1.2.4. *Ranunculus sceleratus*

Other common names for *R. sceleratus* are celery-leaved buttercup and cursed crowfoot. This plant is generally an annual although it occasionally survives a winter.

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[18] Frohne, Pfänder (1997)

[19] Cooper,Johnson (1988)

[20] Cooper, Johnson (1988)

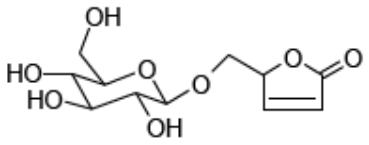
[21] Habermehl, Ziemer (1999)

The plant has fibrous roots and a stout, erect, hollow stem which grows up to 60 cm high. The lower leaves have long stalks with three deeply segmented, toothed lobes near the top, giving them a superficial resemblance to edible celery, although the stalks are thinner. The stem-leaves have short stalks and are divided into narrower segments. This is reputed to be the most poisonous *Ranunculus* species.[22]

1.3. *Ranunculus* – compounds and pharmacological effects

Ranunculus contains the lactones Protoanemonin, Ranunculin and Anemonin. These ingredients also occur in *Anemone*, *Pulsatilla*, *Clematis* and *Helleborus*.[23] Intoxications in humans are rather rare, but the handling of fresh cut plants may cause contact dermatitis. This local irritation has been used in former times for the removal of warts.[24]

1.3.1. Ranunculin



Formula 1: Ranunculin

Ranunculin is a glycoside that enzymatically hydrolyses into glucose and its toxic metabolite Protoanemonin. This hydrolysis can also occur when leaves are dried, crushed or bruised or by alkaline hydrolysis and steam distillation. [25]

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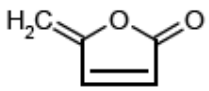
[22] Cooper, Johnson (1988)

[23] Frohne, Pfänder (1997)

[24] Habermehl, Ziemer (1999)

[25] Martz (2008)

1.3.2. Protoanemonin



Formula 2: Protoanemonin

Protoanemonin is a volatile, oily, irritant substance. Protoanemonin has high affinity for SH groups. Its toxic effect as a sub-epidermal vesicant is possibly caused by inactivation of enzymes containing SH groups.[26]

The clinical signs of Protoanemonin poisoning are similar in all animal species. In early stages, salivation, inflammation of the mouth and abdominal pain occur. They may be followed by severe ulcerations of the mouth and damage to the digestive and urinary systems. Colored diarrhea and dark or blood stained urine are produced and at this stage the animals have an unsteady gait, particularly of the hind legs; vision is often impaired or lost. Convulsions usually precede death, although fatal poisoning is rare.[27] In contact with the skin, it produces sub-epidermal disjunction and bulla formation by disruption of sulphur bridges.[28]

Protoanemonin is not stable and dimerizes into Anemonin when the plant is dried. Therefore it is harmless to feed hay containing *Ranunculus*.[29] Protoanemonin has also been shown to possess fungicidal[30,31], antimicrobial[32] and antimutagenic[33] properties.

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[26] Frohne, Pfänder (1997)

[27] Cooper, Johnson (1988)

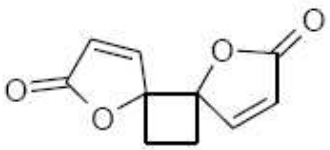
[28] Karaca et al. (2005)

[29] Habermehl, Ziemer (1999)

[30] Misra, Dixit (1980)

[31] Martín et al. (1990)

1.3.3. Anemonin



Formula 3: Anemonin

Anemonin has shown fungicidal (but it is not as potent as its precursor Protoanemonin)[34] and antispasmodic[35] properties. Anemonin is able to modulate the expression of iNOS and therefore inhibit NO production. This could be responsible for its anti-inflammatory effects.[36] Furthermore it inhibits melanin synthesis in human melanocytes[37]. Both Anemonin and Protoanemonin participate in the sedating effect.[38]

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[32] Tocan, Baron (1969)

[33] Minakata et al. (1983)

[34] Misra, Dixit (1980)

[35] Roth et al. (2006)

[36] Lee et al. (2008)

[37] Huang et al. (2008)

[38] Martin et al. (1988)

1.4. ^{1}H-NMR metabolomics

High resolution ^{1}H-NMR spectroscopy with chemometric analysis offers an innovative way to assess whole plant extracts. The potential of metabolomics is currently explored in a wide range of fields including as an efficient method for quality control of phytomedicines. It also provides an important tool for the identification of chemotypes and targets bioactive compounds.[39]

All the chemical components present in a plant extract are viewed simultaneously as a “metabolic fingerprint” in an ^{1}H-NMR spectrum. Such data can be analyzed using different chemometric methods, since the spectra are far too complex to be interpreted visually.[40] The complex “metabolic fingerprint” generated can subsequently be visualised by application of multivariate statistical data analysis in order to reduce the complexity of the data and to detect the pattern of changes relating to environmentally or genetically induced variations in metabolite composition.[41]

Statistical methods which can be used include principal component analysis (PCA) and partial least squares (PLS).

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[39] Cardoso-Taketa et al. (2008)

[40] Rasmussen et al. (2006)

[41] Wang et al. (2004)

1.4.1. Nuclear magnetic resonance (NMR)

Some nuclei carry a total spin (1H, 2H, 13C, 15N...) resulting in a magnetic moment. Only these nuclei can be detected by NMR[42]. In NMR-spectroscopy samples of liquid or solid material are exposed to an external static and homogenous magnetic field B0 The magnetic moments due to spin-1/2 particles can only align parallel or anti-parallel with respect to the external field. For the detection of the NMR signal, a high-frequency magnetic field B1 is applied orthogonal to the external magnetic field for a defined time period. This is called a B1-pulse. Afterwards the magnetization starts to precess around B0.[43] The nuclei release this energy in the form of cosinusoidal oscillation, called the 'free induction decay'. Via a mathematical operation called Fourier-transformation this signal can be transformed into the NMR-spectrum[44].
The resonances are influenced in characteristic ways by the environments of the observed nuclei. In molecules, nuclei are always surrounded by electrons and other atoms. The result of this is that the effective magnetic field B_{eff} at the nucleus is always less than the applied field B_0. The effect, although small, is measurable. It is usually expressed as the σ-value or chemical shift. Before each measurement a reference compound is added to the sample: it is therefore called internal standard. Usually tetramethylsilan (TMS) is used for this purpose. It contains 12 highly shielded protons, which means only a small amount needs to be added, and it is chemically inert and it gives only one sharp peak[45].

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[42] Herzog, Messerschmidt (1995)

[43] Lindon et al. (2007)

[44] Herzog, Messerschmidt (1995)

[45] Friebolin (2005)

1.4.2. Principal Component Analysis (PCA)

Principal Component Analysis (PCA) condenses the multivariate data into a reduced number of principal components that describe the greatest amount of variance in the data. Each PC (principal component) is a linear combination of the original variables whereby each successive PC explains the maximum amount of variance possible in the dataset and each PC is orthogonal to every other PC.

By applying such a technique to the NMR data, similarities and differences between samples can be visualised in simple two- or three-dimensional scores plots.[46] By visual analysis of the scores plots groupings, trends and outliers can be found. Objects or samples that are close to each other in the scores plot have a similar multivariate profile. Conversely, objects that lie far from each other have dissimilar properties.

The loadings plot describes the influence of variables and the relation among them. An important feature is that directions in the score plot correspond to directions in the loading plot, and vice versa.[47]

For example samples with negative scores are associated with negative peaks in the loadings plot.

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[46] Wang et al. (2004)

[47] Lindon et al. (2007)

1.4.3. Regression

Partial least squares (PLS) is a method for relating two data tables. The Y data table can be both quantitative (e.g. metal levels, number of EGS cases) and qualitative (e.g. control/EGS samples) data.[48] Interpretation of the relationship between X-data and Y-data is then simplified as this relationship is concentrated on the smallest possible number of components. By looking at the first PLS components it is possible to view main associations between X-variables and Y-variables, and also interrelationships within X-data and within Y-data.

In these studies PLS was used to prove if there is a relation between the variation within the metabolic profiles of the samples (X-data) and the number of EGS cases that occurred at each site (Y-data).

[48] Lindon et al. (2007)

2. Materials and methods

2.1. Materials

2.1.1. Plant material

The plants were collected and frozen afterwards by Dr. Sarah Edwards and Prof. Dr. Michael Heinrich, Centre for Pharmacognosy and Phytotherapy, between April 2007 and March 2009. The samples of plant material can be divided into three groups:

- Control samples from Golder Manor Farm, that were collected at different times of the year (G)
- Control samples from various sites where no cases of Equine Grass Sickness had been reported (C)
- Samples from sites where Equine Grass Sickness cases had occurred (E)

The plant material that was used for the development of the method was collected at Golder Manor Farm, Oxfordshire, by Dr. Sarah Edwards, Centre of Pharmacognosy and Phytotherapy, between May and August 2008.

Table 1: Golder Manor Farm sample data

Name of the sample	Species	Date of collection	Site
G1	*R. repens*	12.11.2007	Golder Manor Farm Oxfordshire
G2	*R. repens*	5.5.2008	Golder Manor Farm Oxfordshire
G3	*R. repens*	23.5.2008	Golder Manor Farm Oxfordshire
G4	*R. repens*	5.6.2008	Golder Manor Farm Oxfordshire
G5	*R. repens*	8.7.2008	Golder Manor Farm Oxfordshire
G6	*R. repens*	22.8.2008	Golder Manor Farm Oxfordshire
G7	*R. repens*	28.1.2009	Golder Manor Farm Oxfordshire
G8	*R. repens*	20.2.2009	Golder Manor Farm Oxfordshire
G9	*R. repens*	6.3.2009	Golder Manor Farm Oxfordshire
G10	*R. repens*	12.3.2009	Golder Manor Farm Oxfordshire

Table 2: Control sample data

Number of the sample	Species	Date of collection	Site
C1	*R. acris*	28.11.2007	London Equestrian Centre London
C7	*R. repens*	11.6.2008	Swarebrook Great Milton Oxfordshire
C8	*R. acris*	11.6.2008	Swarebrook Great Milton Oxfordshire
C9	*R. repens*	8.7.2008	Overstone Grange Northamptonshire
C10	*R. repens*	15.7.2008	East Bog Farm Angus
C11	*R. repens*	23.7.2008	Checkendon Equestrian Centre Berkshire
C12	*R. acris*	23.7.2008	Checkendon Equestrian Centre Berkshire
C13	*R. acris*	23.7.2008	Wyfold RDA Oxfordshire
C14	*R. repens*	28.7.2008	Town Farm Buckinghamshire
C15	*R. repens*	28.7.2008	Great House Farm Hertfordshire
C16	*R. acris*	28.7.2008	Town Farm Buckinghamshire
C17	*R. repens*	29.8.2008	Potter's Field Buckinghamshire

Table 3: Equine Grass Sickness sample data

Sample number	Species	Number of cases	Date of last case	Date of collection	Site
E1	*R. acris*	1	4.4.2007	15.5.2007	Culbeg Farm Stirlingshire
E2	*R. acris*	1	4.4.2007	15.5.2007	Culbeg Farm Stirlingshire
E3	*R. repens*	2	11.5.2005	17.5.2007	Burgie House Morayshire
E5	*R. repens*	5	19.5.2007	25.5.2007	Home Farm Northamptonshire
E6	*R. repens*	5	19.5.2007	25.5.2007	Home Farm Northamptonshire
E7	*R. acris/ R. repens*	4	22.5.2007	5.6.2007	Bransby Rest Home Lincolnshire
E8	*R. repens*	1	23.4.2007	12.6.2007	Middle of the World Farm Hampshire
E9	*R. acris*	4	9.7.2007	30.7.2007	Catlip's Farm Hertfordshire
E10	*R. repens*	4	13.8.2007	20.8.2007	Poplar's Riding Centre Buckinghamshire
E11	*R. bulbosus*	4	13. 8. 2007	20.8.2007	Poplar's Riding Centre Buckinghamshire
E12	*R. repens*	2	2.12.2007	13.12.2007	Light Ash Farm Lancashire
E13	*R. repens*	2	10.12.2007	29.1.2008	Hendaffrn Powys
E14	*R. bulbosus*	2	8.5.2008	15.5.2008	Willow Lodge Hertfordshire
E15	*R. bulbosus*	2	8.5.2008	15.5.2008	Willow Lodge Hertfordshire
E16	*R. repens*	2	8.5.2008	15.5.2008	Willow Lodge

					Hertfordshire
E17	*R. repens*	1	6.5.2008	19.5.2008	Wargrave Manor Berkshire
E18	*R. repens*	1	6.5.2008	19.5.2008	Wargrave Manor Berkshire
E19	*R. repens*	1	17.7.2008	28.7.2008	Hillcrest Farm Suffolk

2.1.2. Analytical apparatus

^{1}H-NMR experiments (400 MHz) were conducted on a Bruker Avance instrument. The NMR data was processed using Topspin software. Amix viewer was used to transform the NMR spectra into a data table. The data was normalized in Excel. PCA and PLS were performed using Unscrambler.

2.1.3. Chemicals

Methanol, hexane, ethyl acetate and chloroform (all HPLC grade) were obtained from Fisher Scientific, UK. Deionised water was produced with an Elix from Millipore, France. Deuterated chloroform (99.8%) and deuterated Methanol (99.8%) were obtained from Cambridge Isotope Laboratories, Inc., Cambridge, UK. Deuterium oxide (99.9%) was obtained from GOSS Scientific Instruments Ltd., Cambridge, UK. 3-(Trimethylsilyl) propionic-2,2,3,3-d4 acid ,sodium salt (98%) and tetramethylsilane (99.9%) were obtained from Sigma-Aldrich, Germany.

2.2. Methods

2.2.1. Extraction of the plant material

In order to obtain a broad range of differing polarity compounds and a high Ranunculin yield a method similar to the one developed by Bai et al.[49] was used. All samples were extracted using the same extraction method. All samples were extracted in a triplicate.

The frozen plant material (aerial parts) was lyophilized for about 24 hours and was afterwards ground in a mortar. The powder was put into a beaker for three hours to soak with methanol, and then put into a column (diameter 1.5 cm) and covered with methanol over night. The following day, the plant material was extracted with methanol for six hours. The extract was dried under reduced pressure, then transferred into a vial and dried under nitrogen. The dried extract was stored in a freezer until further sample preparation.

Sample data that was collected during the extraction process can be found in the appendix.

[49] Bai et al.(1996)

2.2.2. Fractionation of the extracts

2.2.2.1. Reasons for fractionating the extracts

Crude methanol extracts consist of hundreds of metabolites within a wide range of polarities and have only low contents of Ranunculin and its metabolites. Due to this it is difficult to interpret their NMR spectra (see Fig. 3) and PCA results. In order to obtain extracts with a higher concentration of toxic metabolites the crude methanol extracts were partitioned between solvents with different polarities. The obtained fractions were analysed using ^{1}H-NMR. The ^{1}H-NMR spectra of each fraction was examined for the existence of toxic lactones such as Ranuculin, Ranunculin-aglycon, Protoanemonin or Anemonin by comparing them with spectral data of the pure compounds.

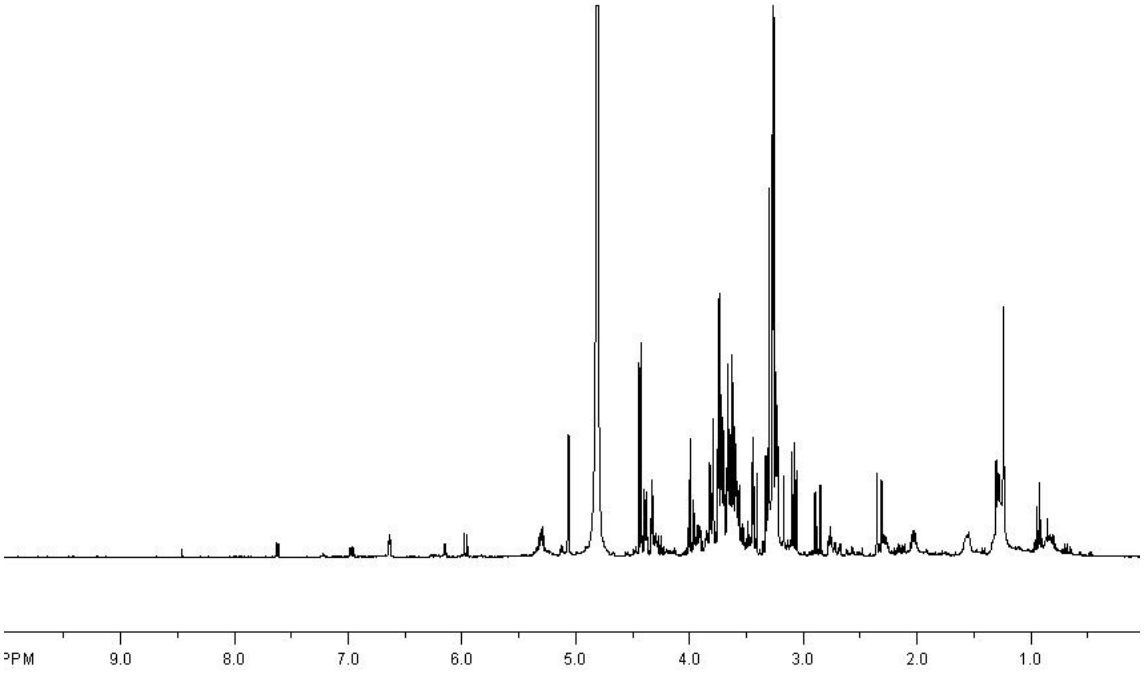
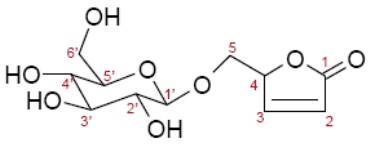


Fig. 3: ^{1}H-NMR of a crude methanol extract in MeOD

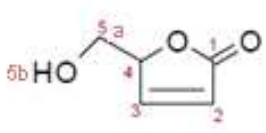
2.2.2.2. NMR data of Ranunculin and its metabolites

Table 4: ^{1}H-NMR data of Ranunculin in MeOD[50]



Position	δH	multiplicity	J
2	6.19	dd	2.2 Hz
			5.6 Hz
3	7.74	dd	1.4 Hz
			6.0 Hz
4	5.33	m	
5	4.11	m	
	3.88	m	
1'	4.32	dd	7.6 Hz
2'	3.17	m	
3'	3.35	dd	1.6 Hz
4'	3.27	dd	0.8 Hz
			5.2 Hz
5'	3.66	m	
6'	3.84	m	
	3.31	m	

Table 5: ^{1}H-NMR data of the aglycon in $CDCl_3$[51]

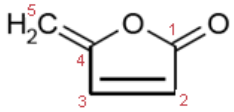


Position	δH	multiplicity	J
2	6.22	dd	2.1 Hz
			5.8 Hz
3	7.48	dd	1.5 Hz
			5.8 Hz
4	5.16	m	
5a	4.00	dd	3.4 Hz
			12.1 Hz
5b	3.80	dd	5.1 Hz
			12.1 Hz

[50] Martz (2008)

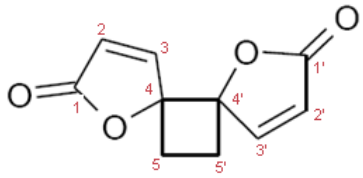
[51] Bai et al. (1996)

Table 6: ^{1}H-NMR data of the Protoanemonin in $CDCl_3$[52]



Position	δH	multiplicity	J
2	6.38	dd	5.6 Hz 0.7 Hz
3	7.72	dd	1.9 Hz 5.6 Hz
5a	5.08	dd	2.8 Hz 0.7 Hz
5b	5.28	dd	2.8 Hz 1.9 Hz

Table 7: ^{1}H-NMR data of the Anemonin in MeOD[53]



Position	δH	multiplicity	J
2 2’	6.18	d	5.3 Hz
3 3’	8.08	d	5.3 Hz
5a 5a’	2.61	m	
5b 5b’	5.35	m	

[52] Blasco et al. (1995)

[53] Huang et al. (2008)

2.2.2.3. Experiments to fractionate the extracts

- **Method 1**

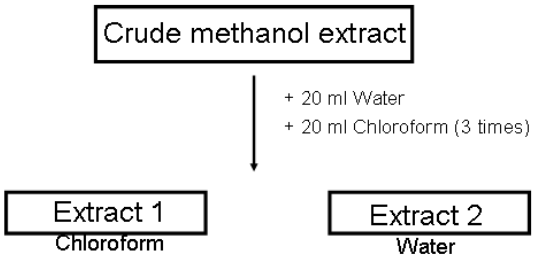


Fig. 4: Schematic diagram of method 1

The dried methanol extract was partitioned in a separating funnel between 20 ml of water and 20 ml of chloroform three times. The chloroform layers (extract 1) were dried under reduced pressure and with nitrogen. Afterwards the chloroform extract was lyophilized, dissolved in $CDCl_3$ (10 mg/ml) and analysed with ^{1}H-NMR (Fig. 5).

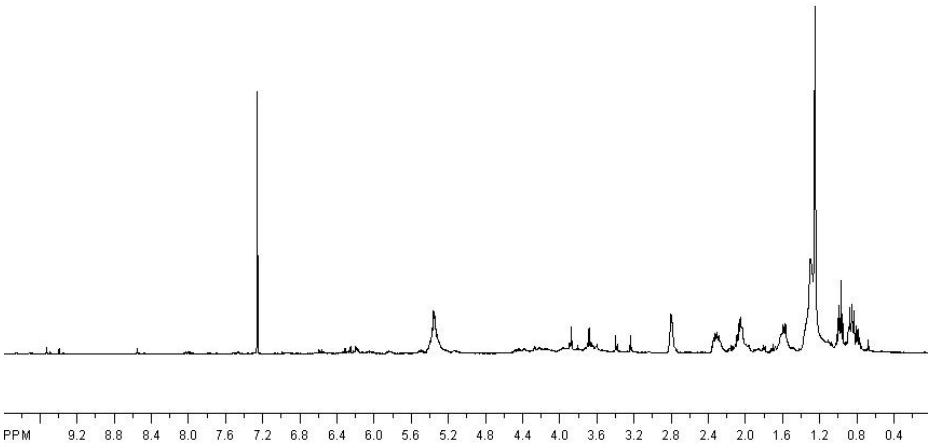


Fig. 5: ^{1}H-NMR of an example of extract 1 (chloroform) in $CDCl_3$

The water layer (extract 2) was freeze-dried and dissolved in D_2O. Due to the formation of precipitate it was not possible to analyse the water extract with ^{1}H-NMR.

- **Method 2**

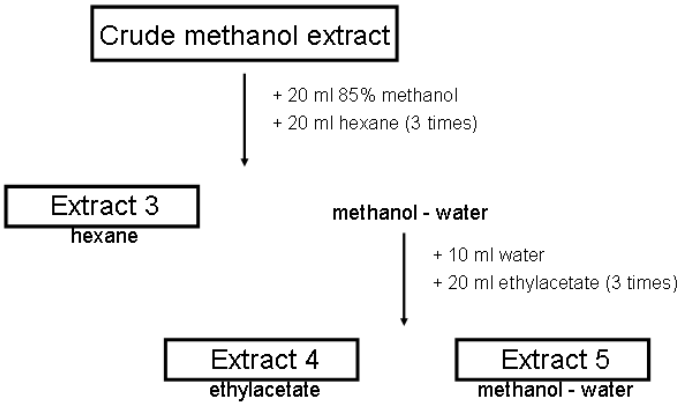


Fig. 6: Schematic diagram of method 2

The dried methanol extract was dissolved in 20ml of 85% methanol and transferred into a separating funnel. The extract was partitioned with 20ml of hexane three times. The hexane layers (extract 3) were dried under reduced pressure and with nitrogen, and were then lyophilized. The dried hexane extract was dissolved in $CDCl_3$ (10mg/ml) and analysed with ^{1}H-NMR (Fig. 7).

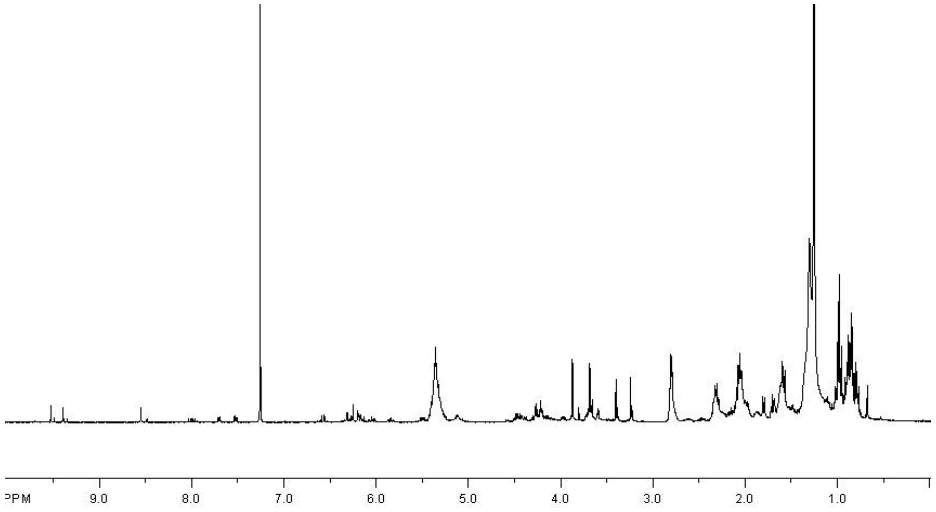


Fig. 7: ^{1}H-NMR of an example of extract 3 (hexane) in $CDCl_3$

The 85% methanol layer was partitioned with 20 ml of ethyl acetate. No separation of layers could be obtained, so an extra 10 ml of water were added to the separation funnel. The ethyl acetate phase (extract 4) was dried under reduced pressure, with nitrogen and was then lyophilized. The dried ethyl acetate extract was dissolved in $CDCl_3$ (10mg/ml) and analysed with ^{1}H-NMR (Fig. 8)

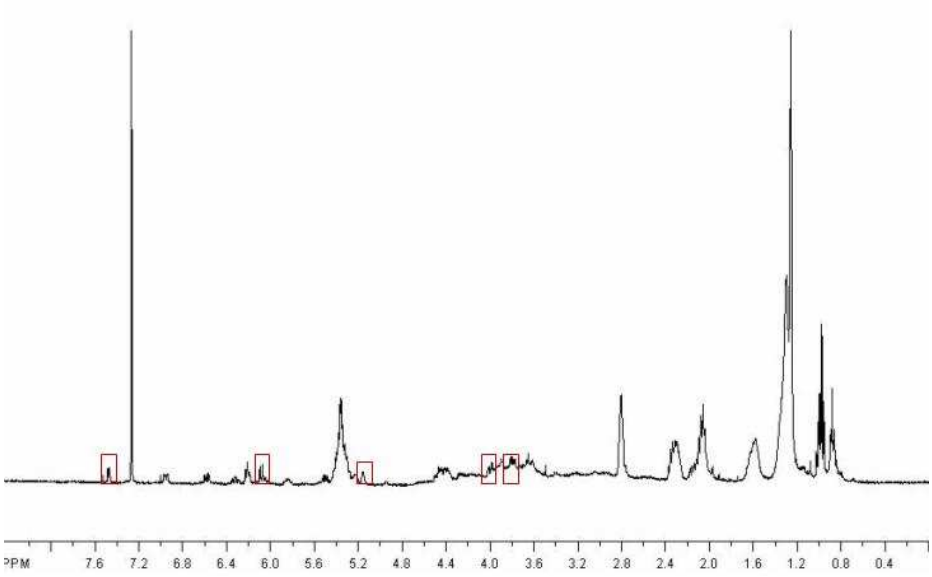


Fig. 8: ^{1}H-NMR of an example of extract 4 (ethyl acetate) in $CDCl_3$

The ^{1}H-NMR spectra of the ethyl acetate fraction showed resonance peaks corresponding to those of the Ranunculin aglycon (for spectral data, see Table 5). The methanol-water layer (extract 5) was dried under reduced pressure, with nitrogen and was then lyophilized. The dried extract was dissolved in MeOD (10mg/ml) but couldn't be analysed because it formed precipitate.

- **Method 3**

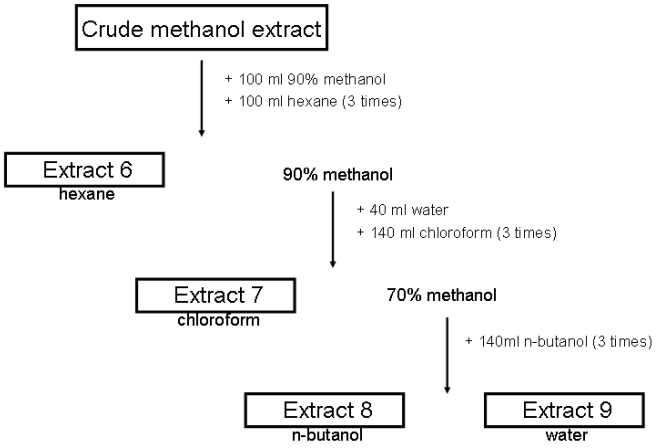


Fig. 9: Schematic diagram of method 3

The dried methanol extract was dissolved in 100 ml of 90% methanol and partitioned with 100ml of hexane three times. The hexane layers (extract 6) were dried under reduced pressure, with nitrogen and were then lyophilized. The hexane extract was dissolved in $CDCl_3$ (10mg/ml) and analysed with ^{1}H-NMR (Fig. 10).

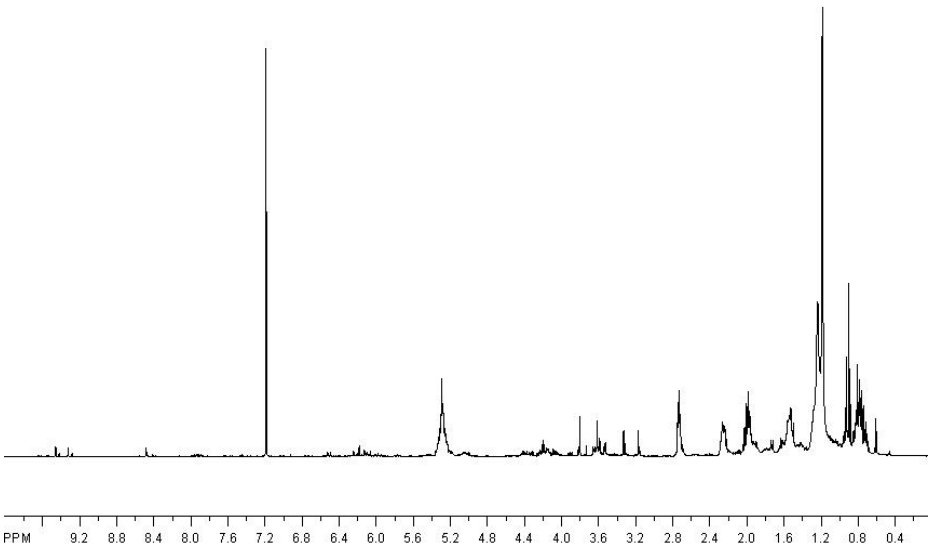


Fig. 10: ^{1}H-NMR of an example of extract 6 (hexane) in $CDCl_3$

40ml of water were added to the methanol layer. The now 70% methanol layer was partitioned with 100 ml chloroform three times. The chloroform layers (extract 7) were dried under reduced pressure, with nitrogen and were then lyophilized. The dried chloroform extract was dissolved in $CDCl_3$ (10mg/ml) and analysed with ^{1}H-NMR (Fig. 11).

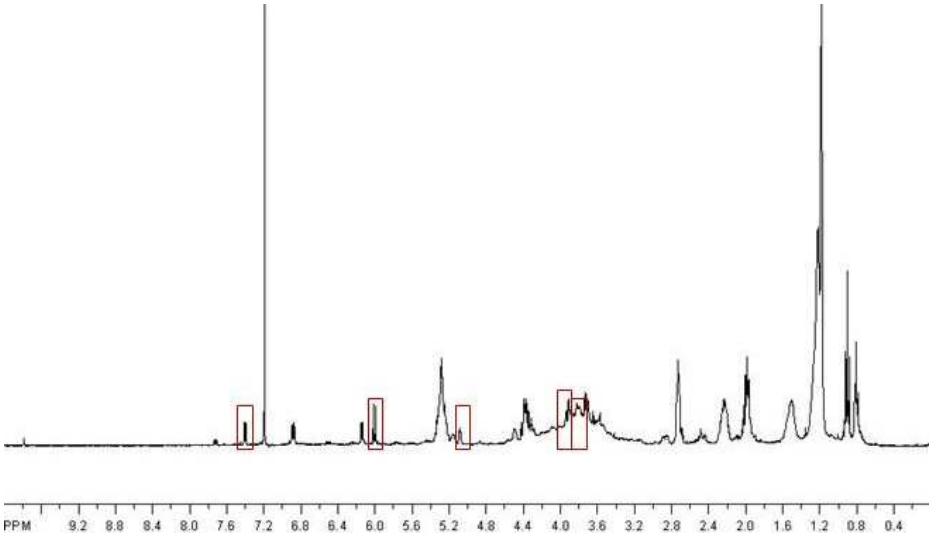


Fig. 11: ^{1}H-NMR of an example of extract 7 (chloroform) in $CDCl_3$

The ^{1}H-NMR spectra of the chloroform fraction showed resonance peaks corresponding to those of the Ranunculin aglycon (for spectral data, see Table 5). The 70% methanol layer was partitioned with butanol three times (1:1). The butanol layers (extract 8) were dried under reduced pressure, with nitrogen and were then lyophilized. The dried butanol extract was dissolved in of $CDCl_3$ (10mg/ml) and analysed with ^{1}H-NMR (Fig. 12).

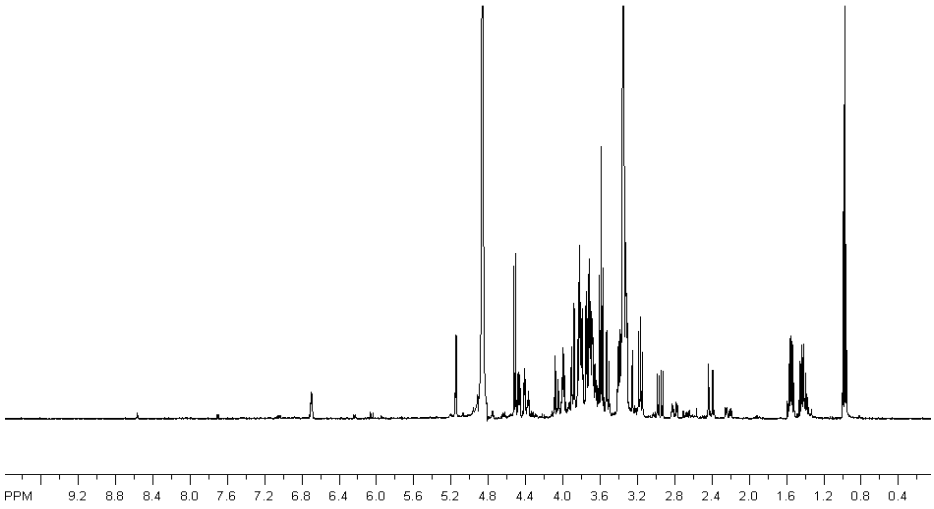


Fig. 12: ^{1}H-NMR of an example of extract 8 (butanol) in MeOD

The remaining water layer (extract 9) was freeze-dried, dissolved in D_2O (10mg/ml) and analysed with ^{1}H-NMR (Fig. 13).

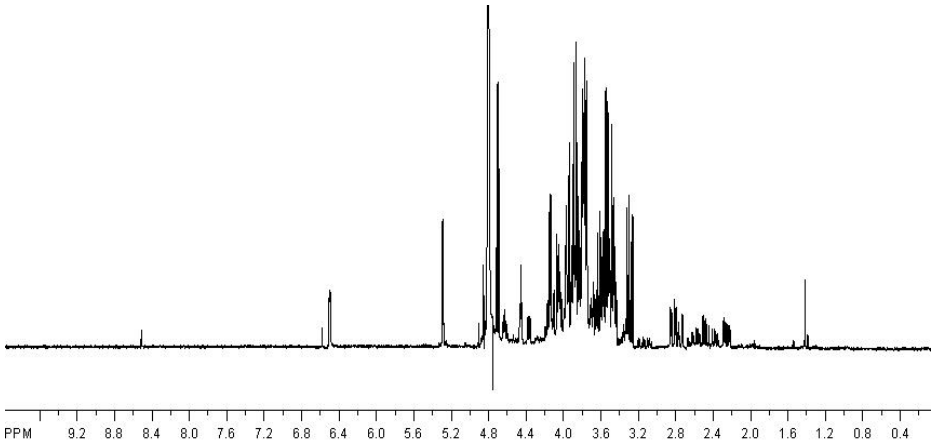


Fig. 13: ^{1}H-NMR of an example of extract 9 (water) in D_2O

- **Conclusions**

Method 3 was the most suitable method because it was possible to analyse all fractions with ^{1}H-NMR. The chloroform fraction had the highest concentration of Ranunculin-aglycon. The NMR spectra of all other fractions did not show resonance

peaks for Ranunculin or any of its metabolites. Because it is possible that not only the concentration of toxins, but the whole range of metabolites in the extract might be involved in EGS, both the crude methanol extracts, as well as their chloroform fractions were analysed.

2.2.3. Sample preparation

- The dried crude methanol extract was dissolved in 50 ml methanol.
- 5 ml of the solution (10% of the total methanol extract) were dried with nitrogen and then lyophilized (**methanol extract**).
- In order to increase the solvent's polarity 5ml of water were added to the remaining 45 ml of the solution. 50 ml of a solution of the extract in 90% methanol were obtained.
- The extract was partitioned with 50 ml of hexane in order to separate apolar compounds (e.g. chlorophyll) from the extract. The hexane extract was then discarded.
- 15 ml of water were added to the 90% methanol fraction in order to increase its polarity. 65 ml of 70% methanol were obtained.
- The 70% methanol fraction was partitioned with 50 ml of chloroform 3 times.
- The chloroform fractions were collected in a round bottom flask and dried under reduced pressure and nitrogen, and the extract was lyophilized (**chloroform extract**).

2.2.4. NMR analysis

The methanol extracts were dissolved in deuterated methanol (MeOD) containing 0.01% TSP to a concentration of 10 mg/ml. The chloroform extracts were dissolved in deuterated chloroform ($CDCl_3$) containing 0.01% TMS to a concentration of 10 mg/ml. The solutions were transferred into 5mm NMR tubes. 1H-NMR spectra were recorded on a Bruker Avance 400 spectrometer. For each sample 128 transients were collected. The spectra were referenced to the internal TMS or TSP.
1H-NMR spectra were corrected for phase and baseline distortions using Topspin.

2.2.5. Data reduction and statistical analysis

Before analysis by multivariate methods the spectra were reduced in complexity by using the “bucketing” function to generate a number of integrated regions of the data set. The spectra in the range of σ0.2 – σ10.5 ppm were divided into 257 regions (“buckets”) of 0.04 ppm using AMIX software and the signal intensity in each region was integrated. Water signals (σ4.6 – σ5.0 ppm in MeOD spectra), residual proton signals corresponding to MeOD (σ3.24 – σ3.4 ppm) and $CDCl_3$ (σ7.2 – σ7.4 ppm) and internal standard peaks (σ-0.2 – σ0.2 ppm) were removed. This data table could then be imported into Microsoft Excel for the addition of labels and normalization. The spectral areas were normalized to the total sum of the spectra integral. PCA and PLS were carried out on the normalized NMR dataset using mean- centered scaling with the software Unscrambler. The results were validated with full cross validation. PCA and PLS summarized the variance in the spectra into 5 principal components.

3. Results

3.1. PCA (Principal Component Analysis)

3.1.1. Comparison of different species

PCA was carried out for methanol extracts on the complete data set (samples from Golder Manor farm (G), samples from other control sites (C) and EGS sites (E)). A five component model explained 86% of the variance, with the first two components explaining 67%. Fig. 14 shows the PCA scores plot (PC1/PC2) for all methanol extracts. A strong classification between two groups of samples is evident in the second principal component (the PC2 direction).

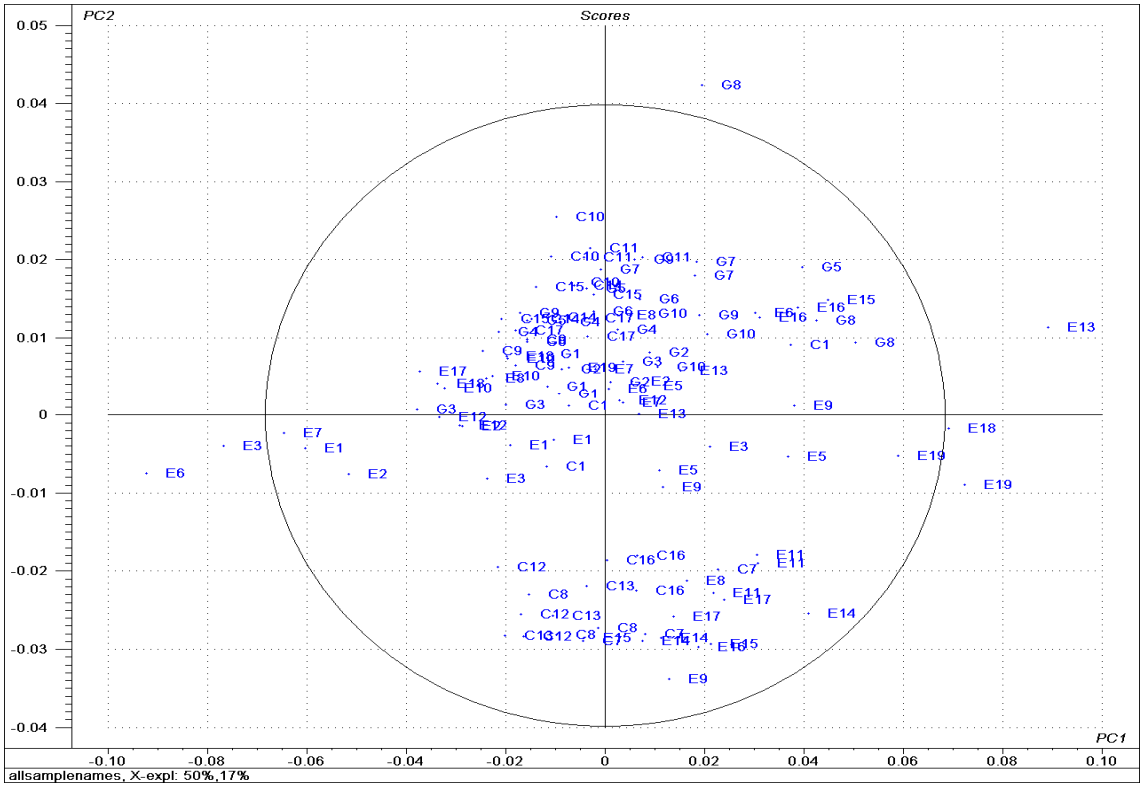


Fig. 14: Scores plot (PC1 (50%) vs. PC2 (17%)) of different methanol extracts

This differentiation is caused by the fact that there are three different *Ranunculus* species: *R. repens, R. acris and R. bulbosus*. With the exception of some outliers (C1, C7, E1, E2 and E17), *R. acris* and *R. bulbosus* samples grouped together, indicating that the chemical composition of these two species is similar (Fig. 15 and Fig. 16).

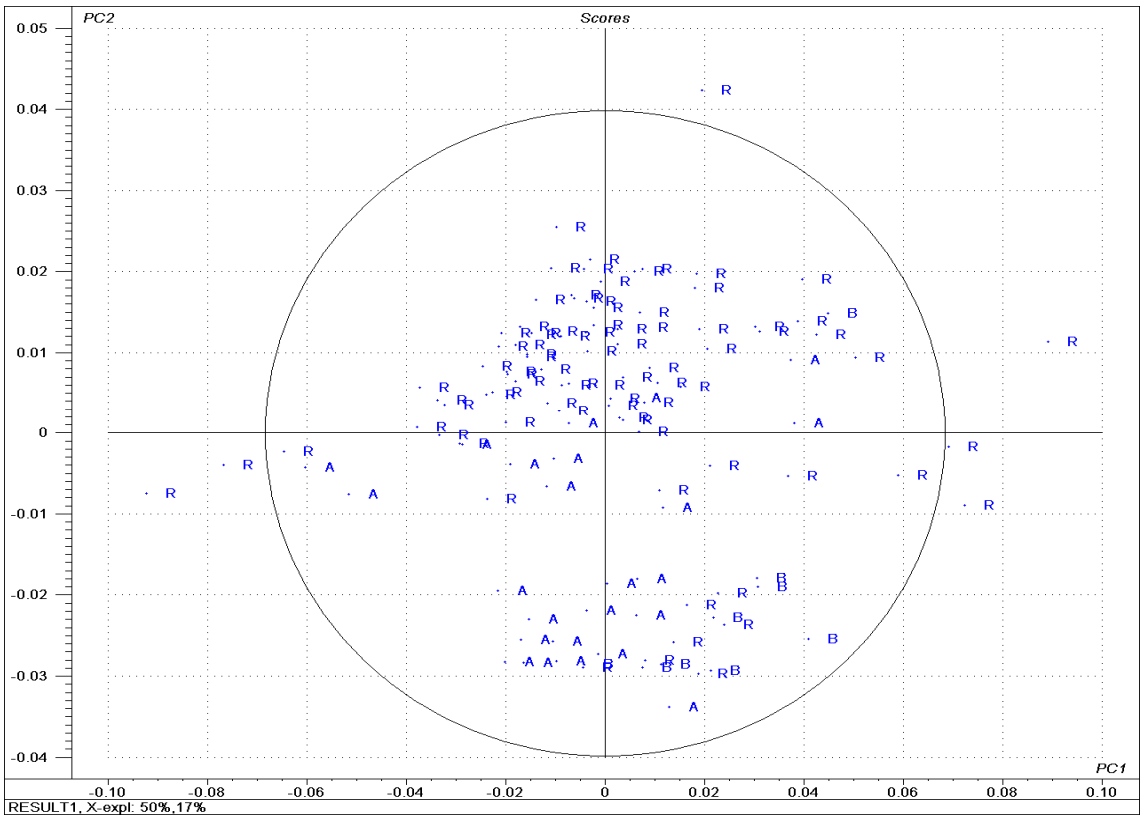


Fig. 15: Scores plot (PC1 (50%) vs. PC2 (17%)) of all methanol extracts. *R. repens* (R), *R. acris* (A), *R. bulbosus* (B).

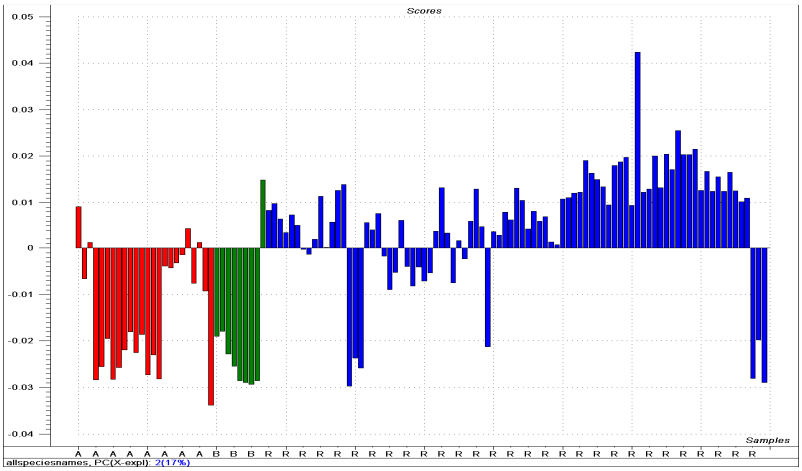


Fig. 16: Scores plot (PC2 (17%)) of all methanol extracts. *R. repens* (blue), *R. acris* (red), *R. bulbosus* (green).

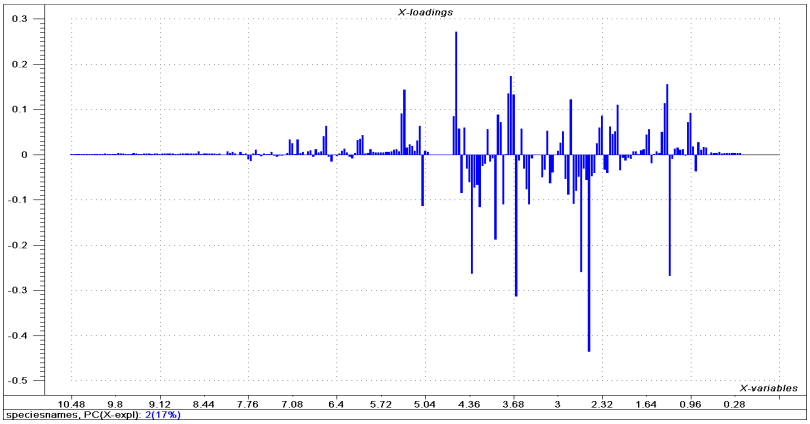


Fig. 17: Loadings plot (PC2 (17%)) of all methanol extracts

Since *R. acris* and *R. bulbosus* can be found in the lower half of the scores plot, the negative loadings are associated with *R. acris* and *R. bulbosus*. Examination of the loadings plot of PC2 (Fig. 17) showed that negative loadings appear mainly in the carbohydrate region (σ4.4 – σ2.3 ppm). On the other hand the positive loadings, which are associated with *R. repens,* appear in the aromatic region (σ8.0 – σ5.5. ppm). There are no visible loadings for Ranunculin aglycon (^{1}H-NMR data in $CDCl_3$: 7.48 (H-3), 6.22 (H-2), 5.16 (H-4), 4.00 (H-5a), 3.80 (H-5b) ppm), therefore Ranunculin aglycon concentration is not one of the main differences between the metabolic profiles of different *Ranunculus* species.

The same experiment was carried out on all chloroform extracts. 86% of the total information could be explained by a five component model, with the first two components explaining 57%. Sample E10 had to be taken out of the analysis, because it was very different to all other samples.

The scores plot of PC1/PC2 showed no clear differentiation between the different species (Fig. 18).

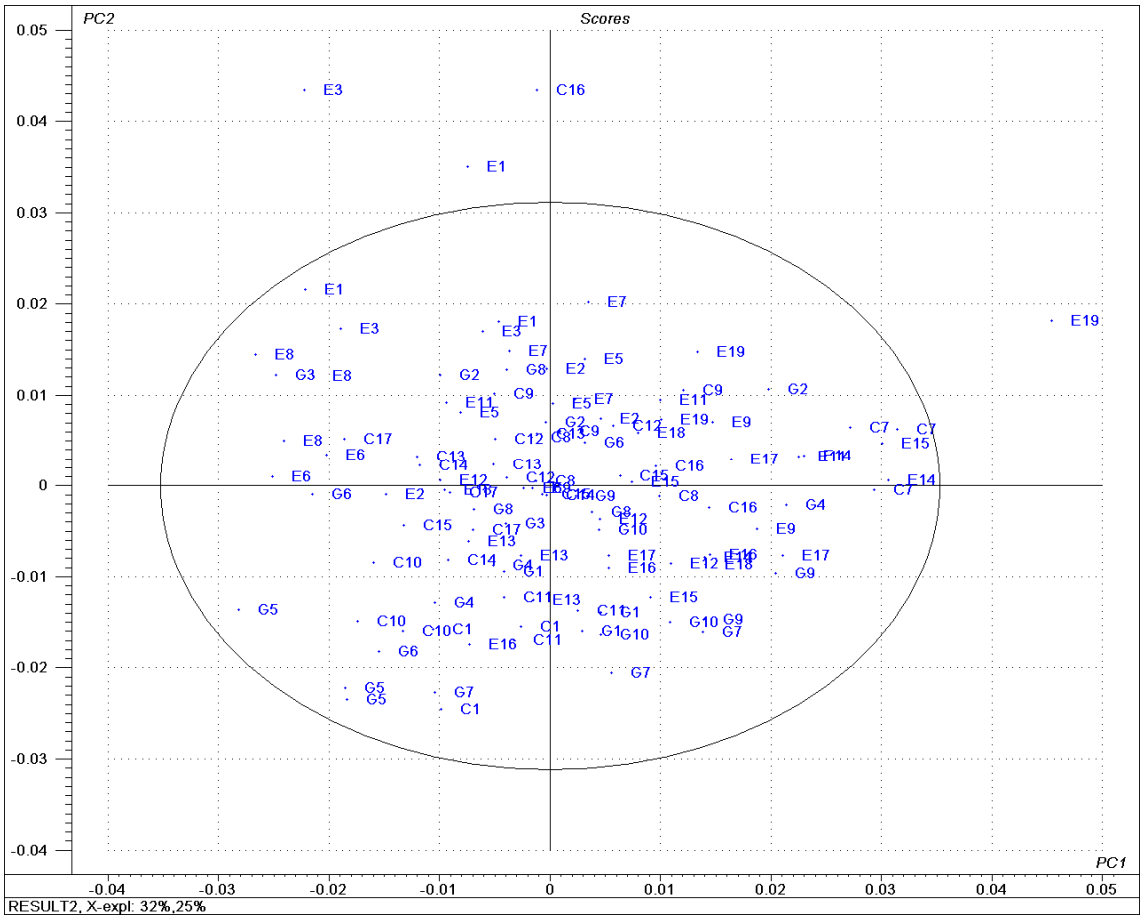


Fig. 18: Scores plot (PC1 (32%) vs. PC2 (25%)) of all chloroform extracts

The loadings plot of PC2 (Fig. 20) showed positive loadings corresponding to the resonance peaks of the Ranunculin aglycon. This showed that the concentration of Ranunculin aglycon is an important characteristic for the differentiation of the chloroform extracts. Therefore it can be estimated that samples with positive PC2 scores (located in the upper half of the scores plot) have higher contents of Ranunculin aglycon. The scores plot of PC2 showed high scores for all *R. acris* samples (C12, C13, C16, E1, E2, E7 and E9), with the exception of sample C1. This indicates that *R. acris* has higher toxin content than *R. repens* or *R. bulbosus.* Although most *R. repens* samples have lower PC2 scores, there are also some samples with very high PC2 scores (E3, E5, E7, E8, E19, C9, G2).

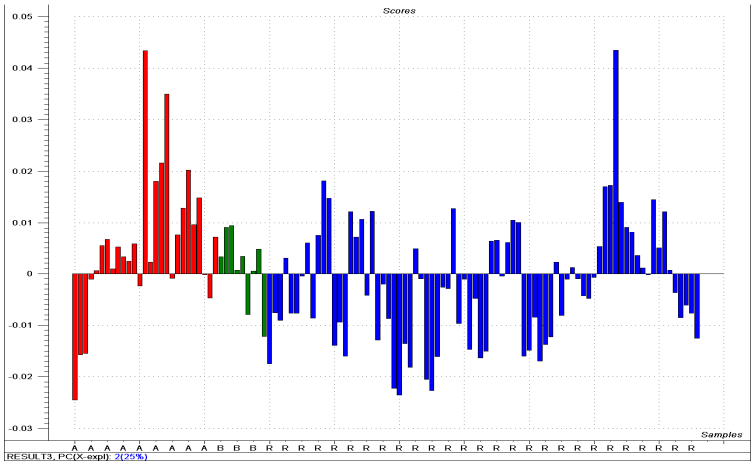


Fig. 19: Scores plot (PC2 (25%)) of all chloroform extracts. *R. repens* (blue), *R. acris* (red), *R. bulbosus* (green).

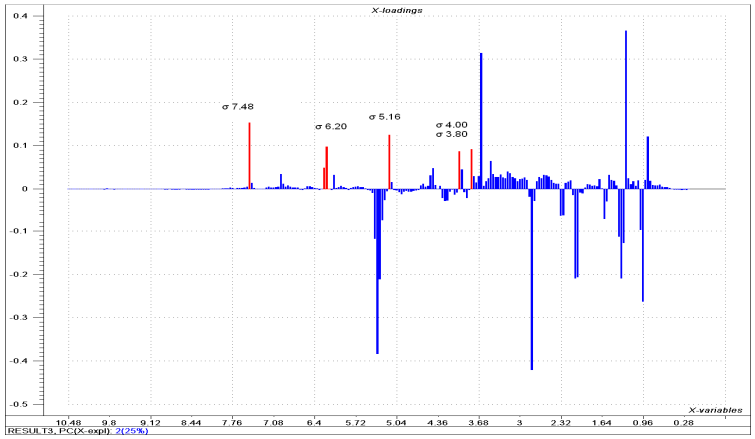


Fig. 20: Loadings plot (PC2 (25%)) of all chloroform extracts. Resonance peaks of Ranunculin aglycon (red).

3.1.2. Comparison of *R. acris* and *R. bulbosus*

Although *R. acris* and *R. bulbosus* appear to have a very similar chemical composition it was possible to detect a difference comparing only extracts of these two species.

Fig. 21 shows the PCA scores plot for methanol extracts of *R. acris* (C1, C8, C12, C13, C16, E1, E2, E7 and E9) and *R. bulbosus* (E11, E14 and E15). A differentiation is evident in the first principal component (PC1). All *R. bulbosus* samples (with the exception of E15/3) group together as a cluster. Sample E9 (*R. acris*) has similar properties as *R. bulbosus* samples.

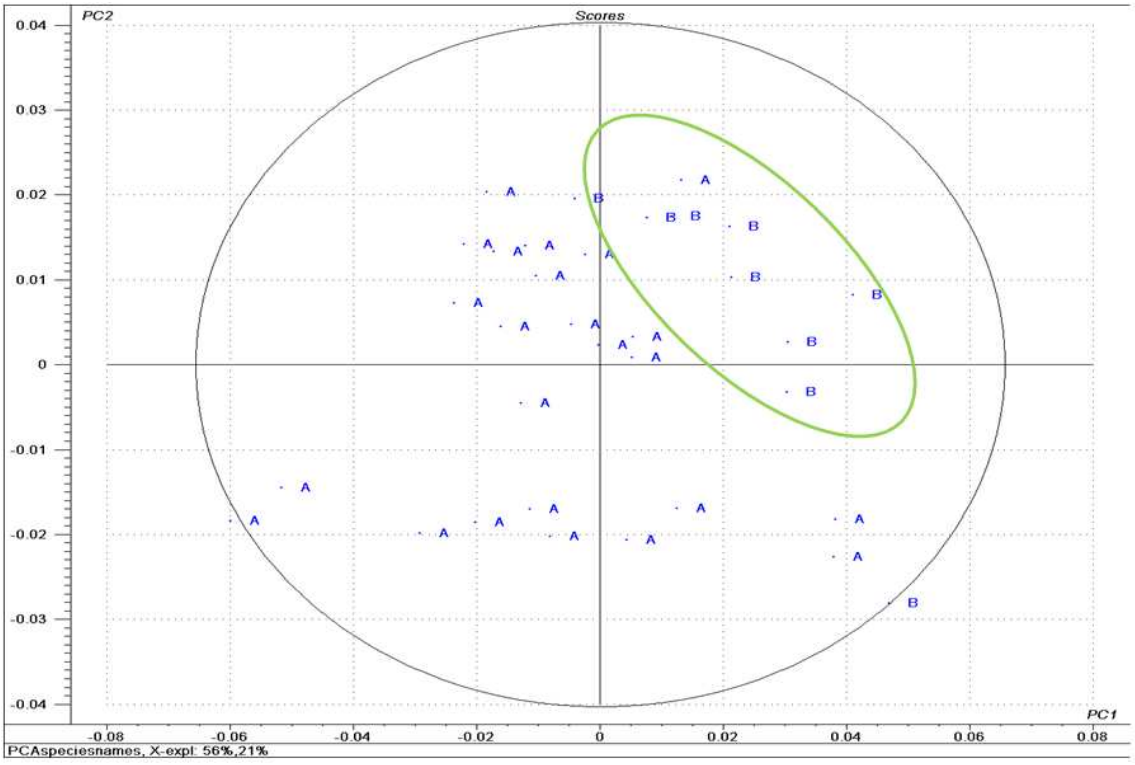


Fig. 21: Scores plot (PC1 (56%) vs. PC2 (21%)) of methanol extracts of *R. acris* (A) and *R. bulbosus* (B).

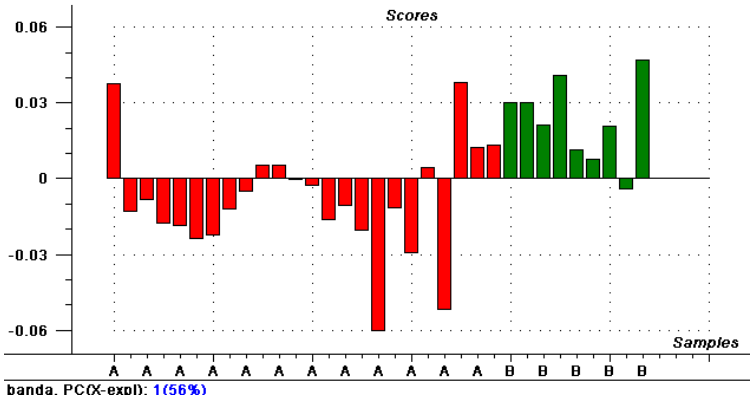


Fig. 22: Scores plot (PC1 (56%)) for methanol extracts of *R. acris* (red) and *R. bulbosus* (green).

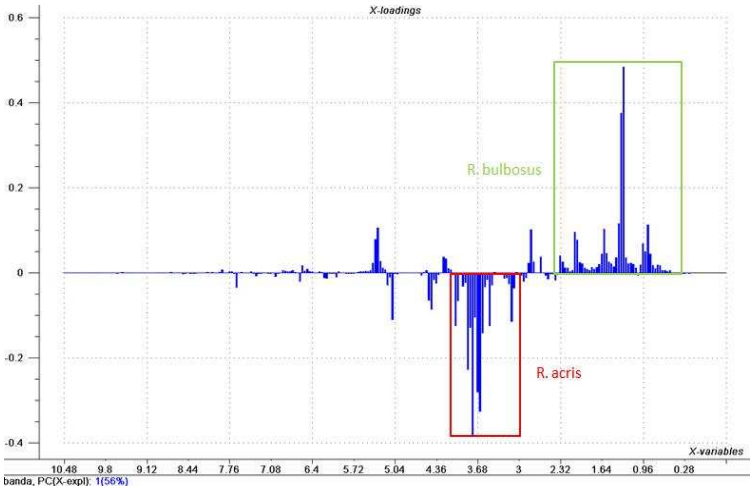


Fig. 23: PCA loadings plot (PC1 (56%)) for methanol extracts of *R. acris* (red) and *R. bulbosus* (green).

The loadings plot of PC1 (Fig. 23) shows clearly that the differentiation of the clusters results from differences in the resonance regions of σ2.5 – σ4.5 ppm and σ0.5 – σ2.5 ppm. Generally negative loadings, which are associated with *R. acris* show high peaks in the carbohydrate region, whereas positive loadings (associated with *R. bulbosus*) appear mainly in the aliphatic region.

3.1.3. Comparison of different seasons

In order to detect changes relating to environmentally or genetically induced variations in the metabolite composition it was necessary to compare *R. repens*

samples only. Without the variation between different species it was possible to find out if the season of sample collection has an impact on the plant's chemical composition. Samples G1, G7, G8, G9, G10, E12 and E13 were collected in winter (between November and March). Samples G2, G3, E3, E5, E6, E16, E17 and E18 were collected in spring (May) and G4, G5, G6, C7, C9, C10, C11, C14, C15, C17, E8, E10 and E19 were collected in summer (between June and August).

The PCA scores plot for methanol extracts (Fig. 24) showed no clustering in the first two principal components, but the scores plot of PC3/PC4 showed a cluster of samples that were collected in winter (with the exception of E12/2 and E13/3). There was no apparent differentiation between samples collected in spring and in summer. This indicates that there are only minor differences between the metabolite composition of plants during their vegetation period and in winter.

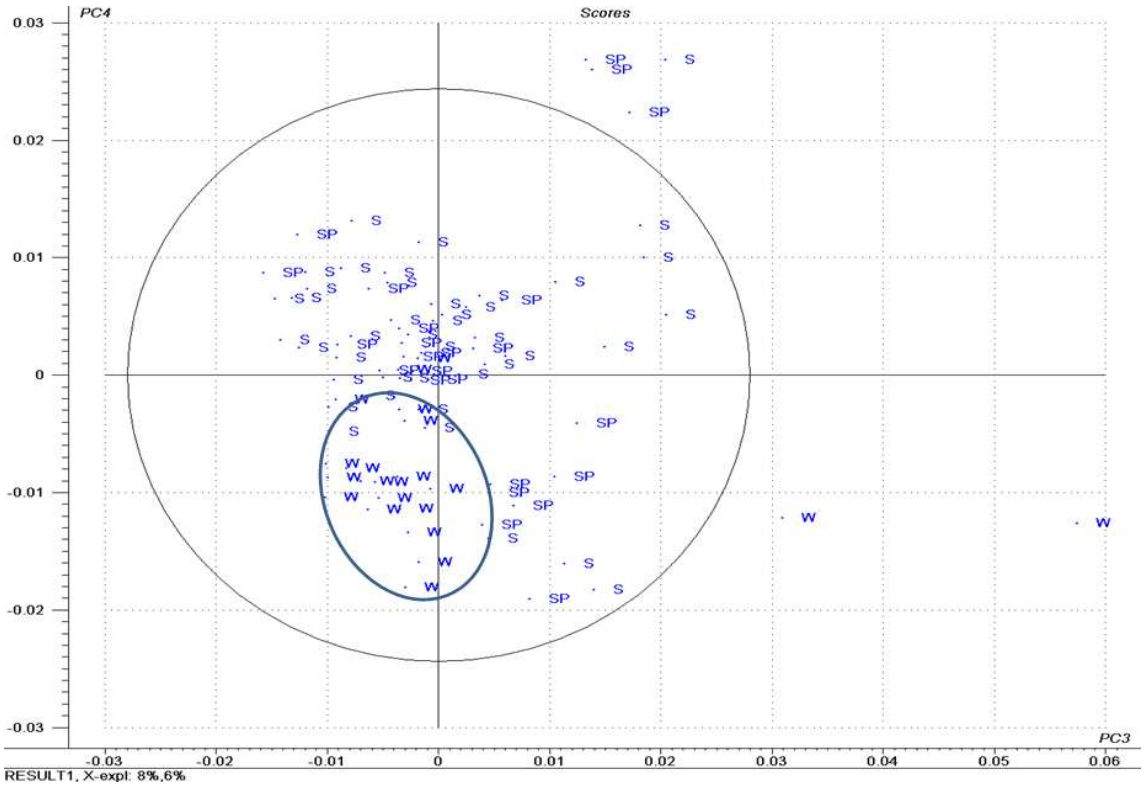


Fig. 24: Scores plot (PC3 (8%)/PC4 (6%)) of *R. repens* methanol extracts. Spring (SP), summer (S), winter (W).

The loadings plot of PC4 (Fig. 26) showed that negative loadings, which are associated with samples collected in winter appear mainly in the aliphatic region (σ2.30 – σ0.00 ppm), whereas positive loadings correspond to peaks in the carbohydrate and aromatic region (σ9.00 – σ2.30 ppm).

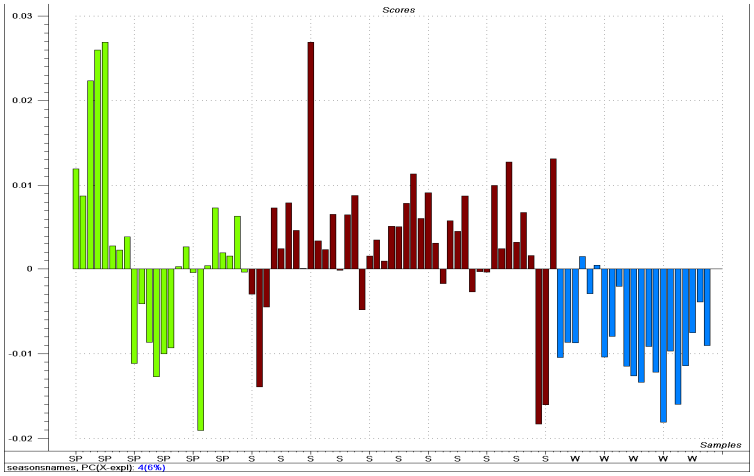


Fig. 25: Scores plot (PC4 (6%)) of *R. repens* methanol extracts. Spring (SP), summer (S), winter (W).

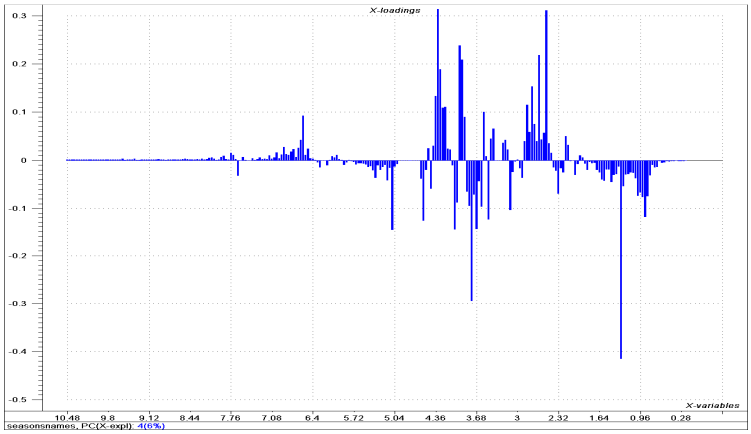


Fig. 26: Loadings plot (PC4 (6%)) of *R. repens* methanol extracts.

PCA was also carried out with all chloroform extracts of *R. repens* samples. The scores plot of PC2/PC3 showed that samples in winter are predominantly located in the right half of the scores plot (Fig. 27). However there are many samples collected in summer and spring with similar properties, so this differentiation is not very specific.

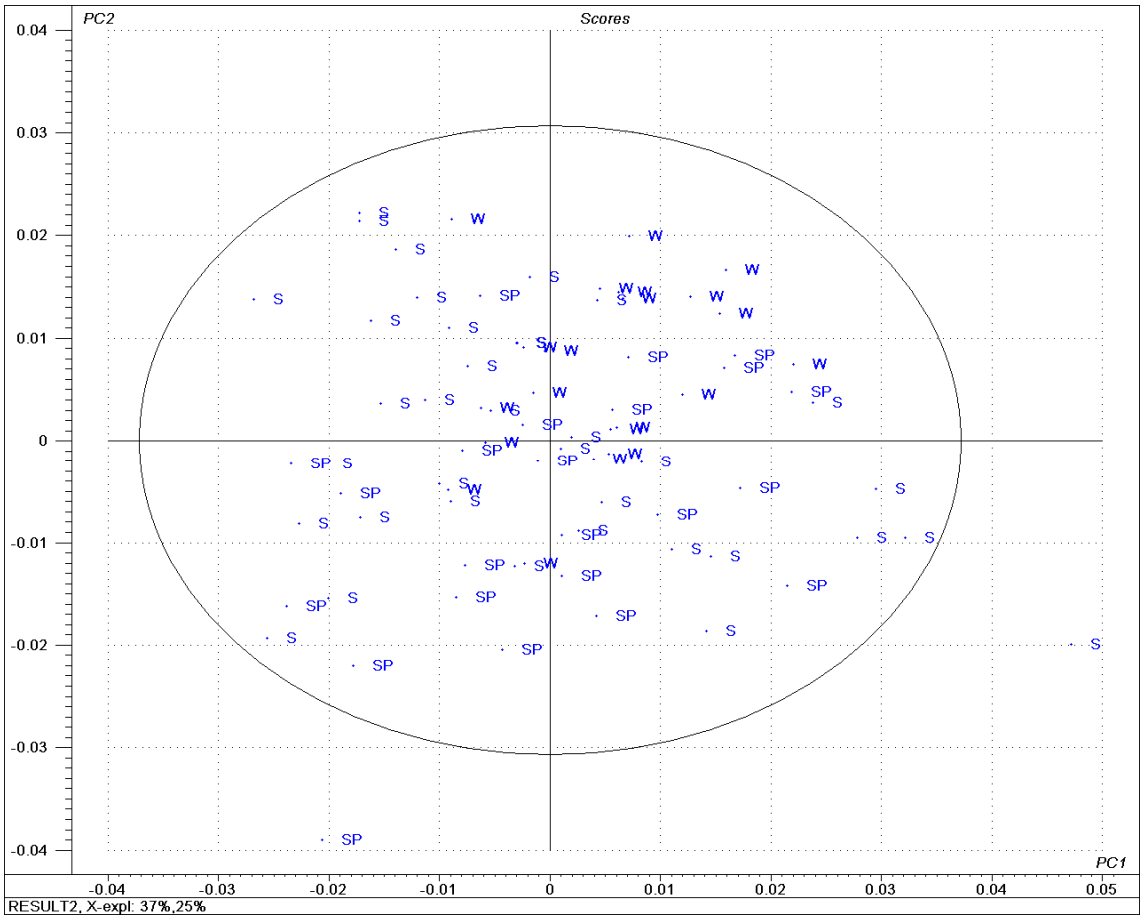


Fig. 27: Scores plot (PC1 (37%) vs. PC2 (25%)) of *R. repens* chloroform extracts. Spring (SP), summer (S), winter (W).

By comparing the PC2 scores plot with its loadings plot (Fig. 28 and Fig. 29), it was possible to show that most samples collected in winter have lower contents of Ranunculin aglycon. On the other hand, most samples collected in spring have negative PC2 scores and therefore high Ranunculin aglycon concentration.

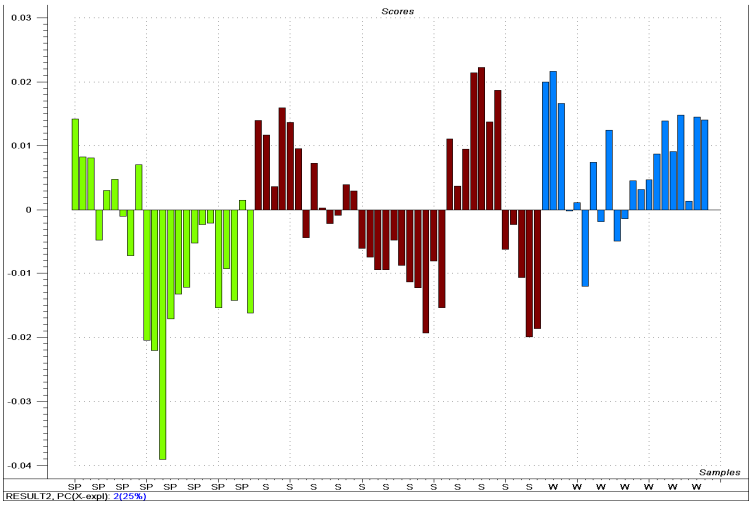


Fig. 28: Scores plot (PC2 (25%)) of *R. repens* chloroform extracts. Spring (SP), summer (S), winter (W).

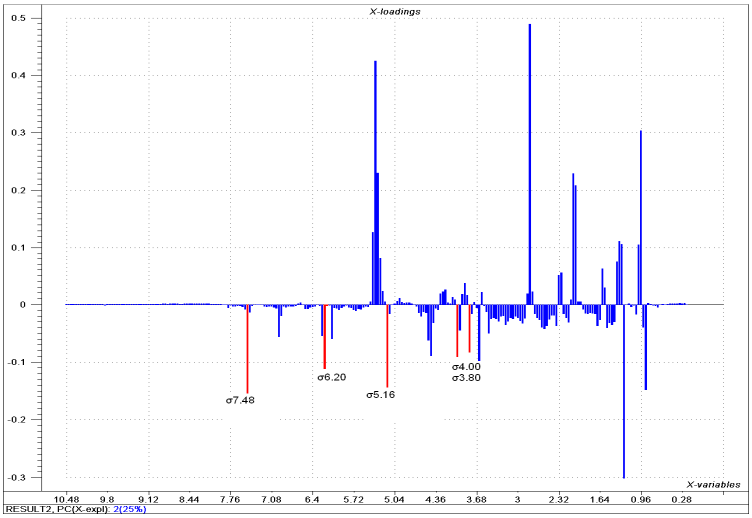


Fig. 29: Loadings plot (PC2 (25%)) of *R. repens* chloroform extracts. Ranunculin aglycon (red).

3.1.4. Samples from only one site

Comparing samples from only one site (Golder Manor Farm) that have been collected at different times of the year, makes it easier to draw conclusions about seasonal differences. In this experiment the impact of environmental factors (such as geological properties or different climates) has been minimized.

Samples collected in winter form a cluster in PC2/PC3. The loadings plot of PC3 showed positive loadings between σ3.2 - σ4.2 ppm and σ 5.2 – σ5.6 ppm which correspond to samples collected in winter. The results showed that samples collected in winter differ from samples collected in spring or summer.

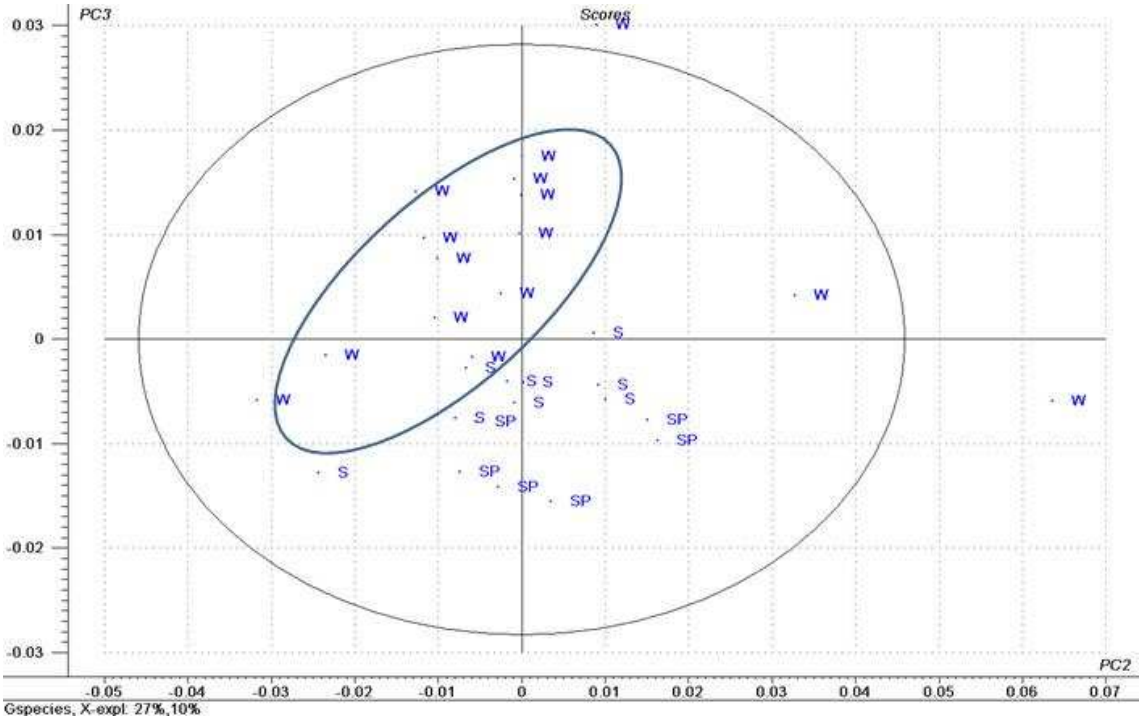


Fig. 30: Scores plot (PC2 (27%) vs. PC3 (10%)) of methanol extracts from Golder Manor Farm. Spring (SP), summer (S), winter (W).

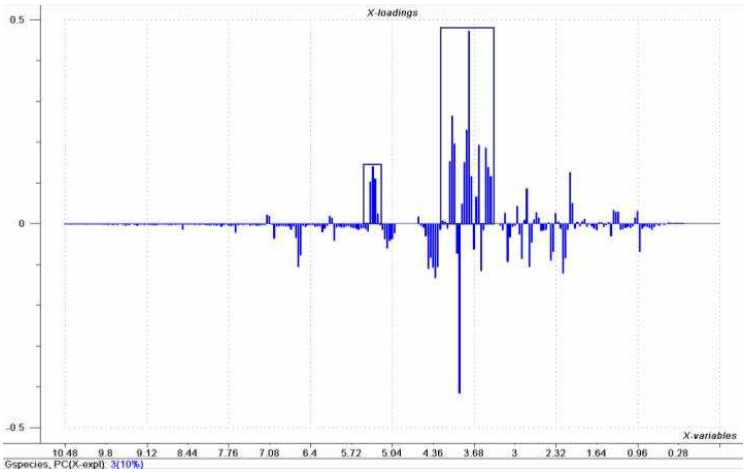


Fig. 31: Loadings plot (PC3 (10%)) of methanol extracts from Golder Manor Farm.

PCA was also carried out on all chloroform extracts from Golder Manor farm. Samples collected in winter were predominantly located in the right half of the scores plot of PC1/PC2 (Fig. 32).

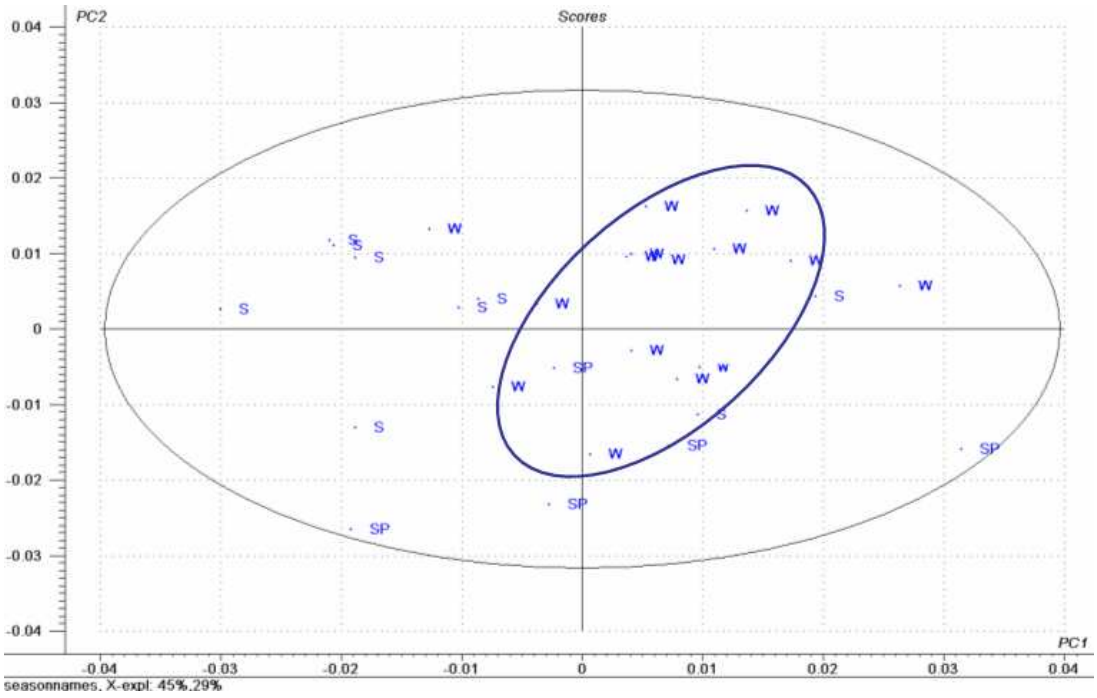


Fig. 32: PCA scores plot (PC1 (45%) vs. PC2 (29%)) of chloroform extracts from Golder Manor Farm. Spring (SP), summer (S), winter (W).

3.1.5. Comparison of EGS and control sites

To evaluate the main purpose of these studies, whether samples from EGS sites are significantly different from samples from control sites, it is important to compare only samples from one species. Therefore PCA was carried out on only *R. repens* methanol extracts from all Equine Grass sickness sites and control sites.

The scores plot of PC1/ PC 2 showed a cluster of control samples (with the exception of C7) whereas there was a higher variation between the EGS samples (Fig. 33, Fig. 34 and Fig. 35).

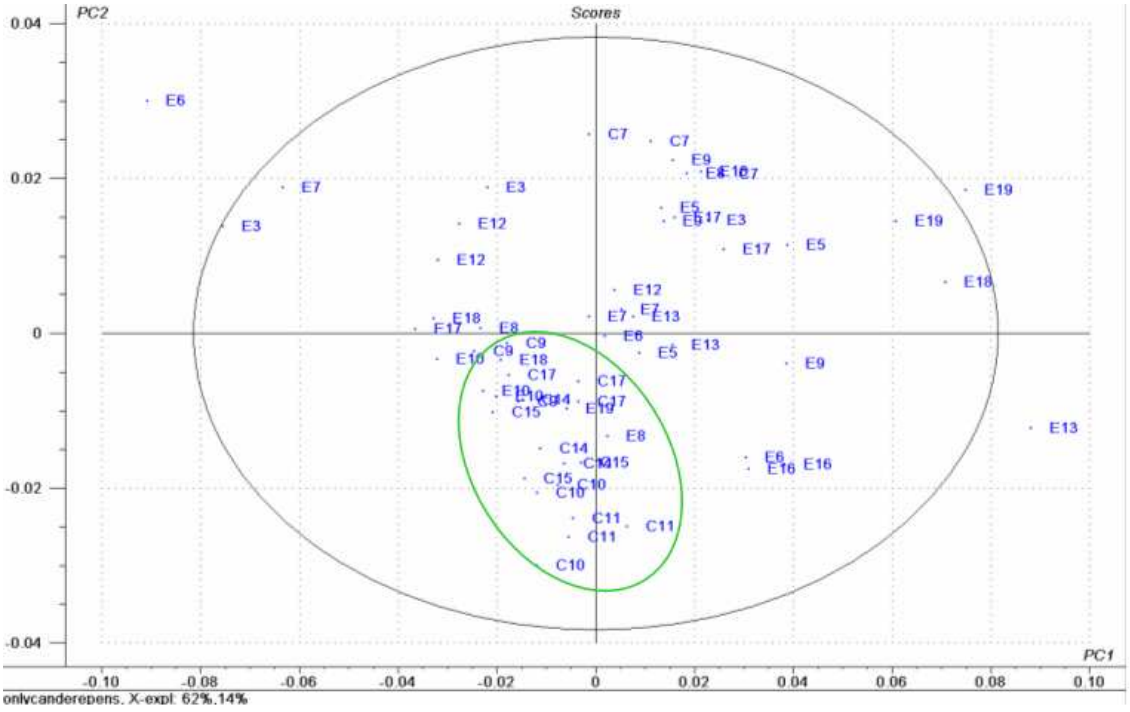


Fig. 33: Scores plot (PC1 (62%) vs. PC2 (14%)) of methanol extracts from control (C) and EGS sites (E).

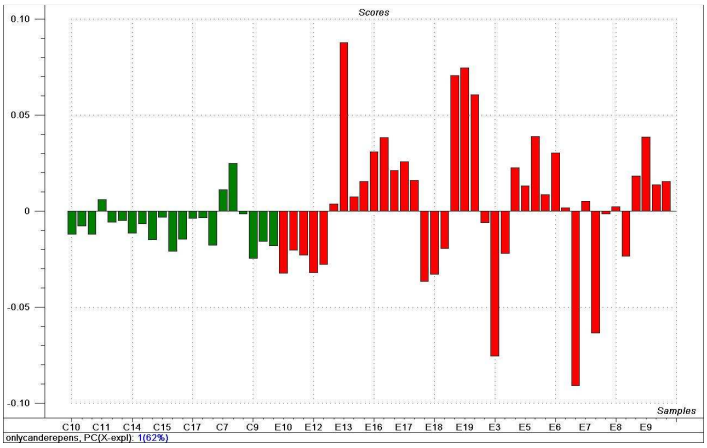


Fig. 34: Scores plot (PC1 (62%)) of methanol extracts from control (C) and EGS sites (E).

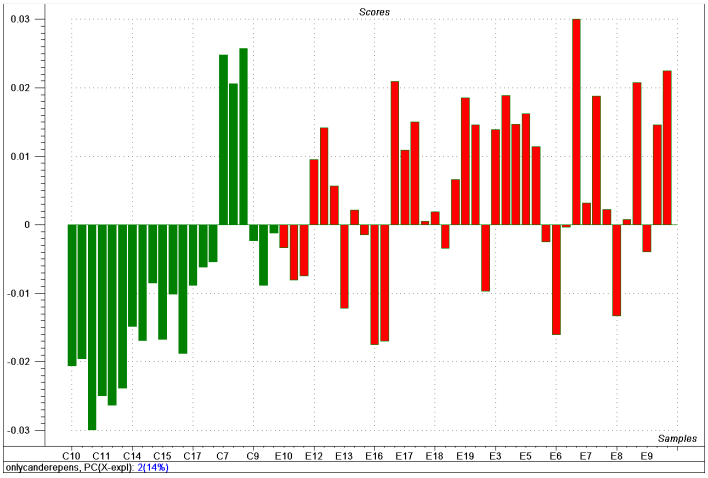


Fig. 35: Scores plot (PC2 (14%)) of methanol extracts from control (C) and EGS sites (E).

The same experiment was carried out on the chloroform extracts. Even though the clustering of control samples is not as evident as for the methanol extracts the overall picture is very similar (Fig. 36). The loadings plot for PC2 (Fig. 37) showed positive loadings corresponding to the resonance peaks of the Ranunculin aglycon. Therefore samples with positive PC2 scores (located in the upper half of the scores plot) have higher contents of Ranunculin aglycon. Most control samples (with the exception of C7 and C9) have low PC2 scores, so their Ranunculin aglycon concentrations are approximately lower.

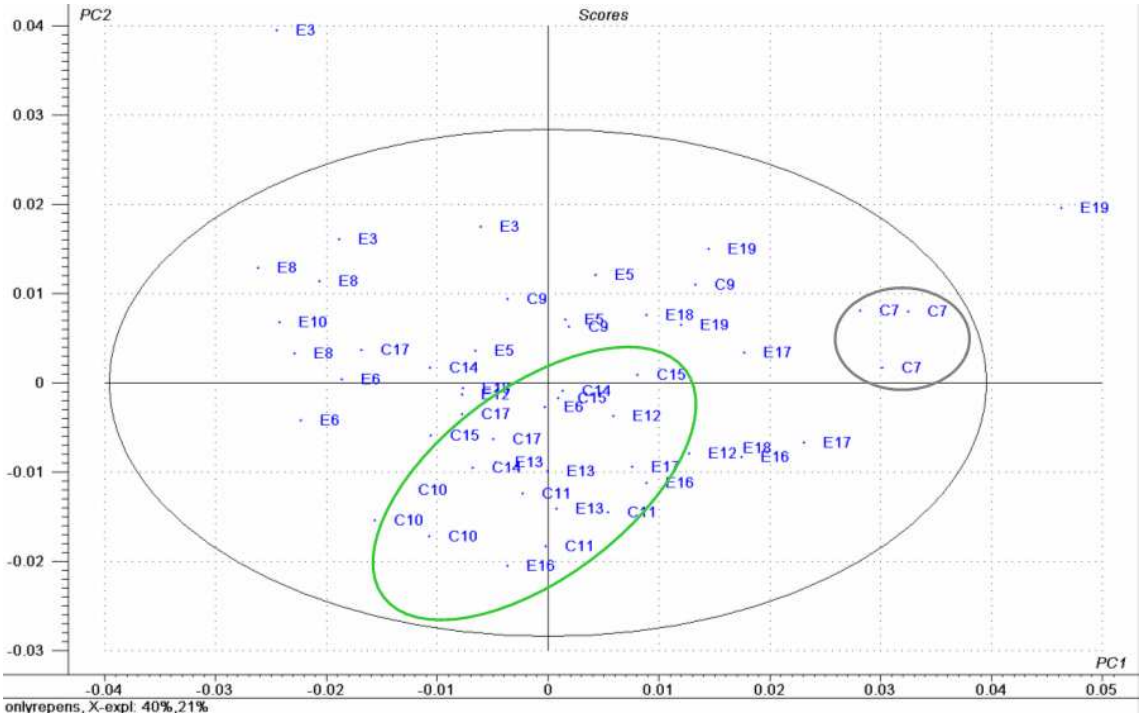


Fig. 36: Scores plot (PC1 (40% vs. PC2 (21%)) of chloroform extracts from control (C) and EGS sites (E).

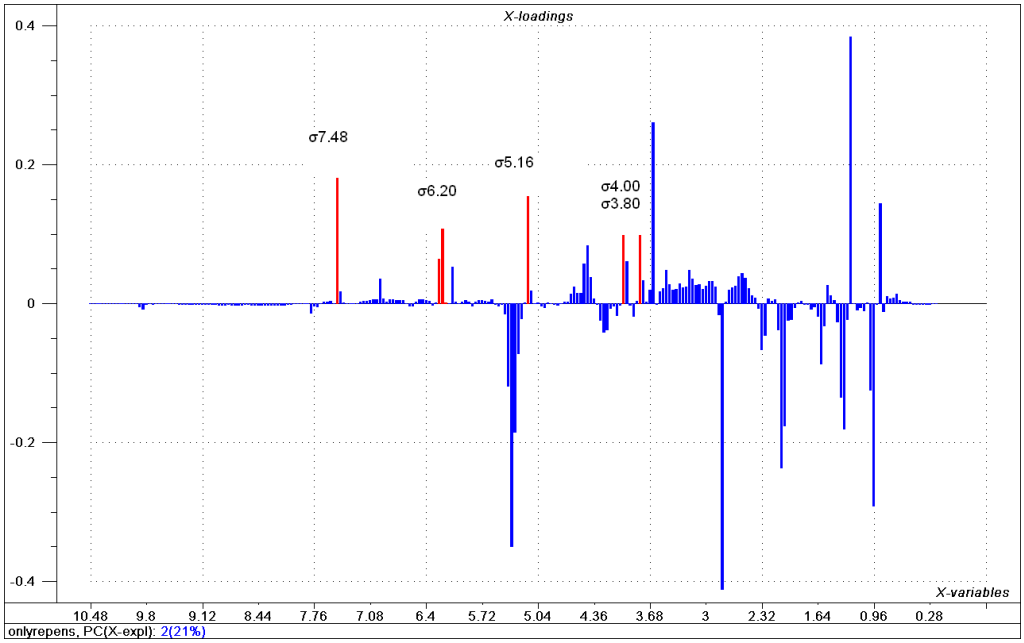


Fig. 37: Loadings plot (PC2 (21%)) of chloroform extracts. Resonance peaks for Ranunculin aglycon (red)

3.2. PLS-DA (partial least squares - discriminant analysis)

PLS-DA was carried out on methanol extracts of *R. repens* samples from Equine Grass Sickness (E) and control sites (C). The y variable was defined as either 0 (no EGS case occurred) or 1 (EGS cases had occurred). A five component model explained 87% of the variance within the x variables (spectra), with the first two components explaining 74%. 45% of this variation could be explained by the y variable (0 or 1).

The scores plot of PC1/2 (Fig. 38) showed a cluster of all control sites with the exception of C7.

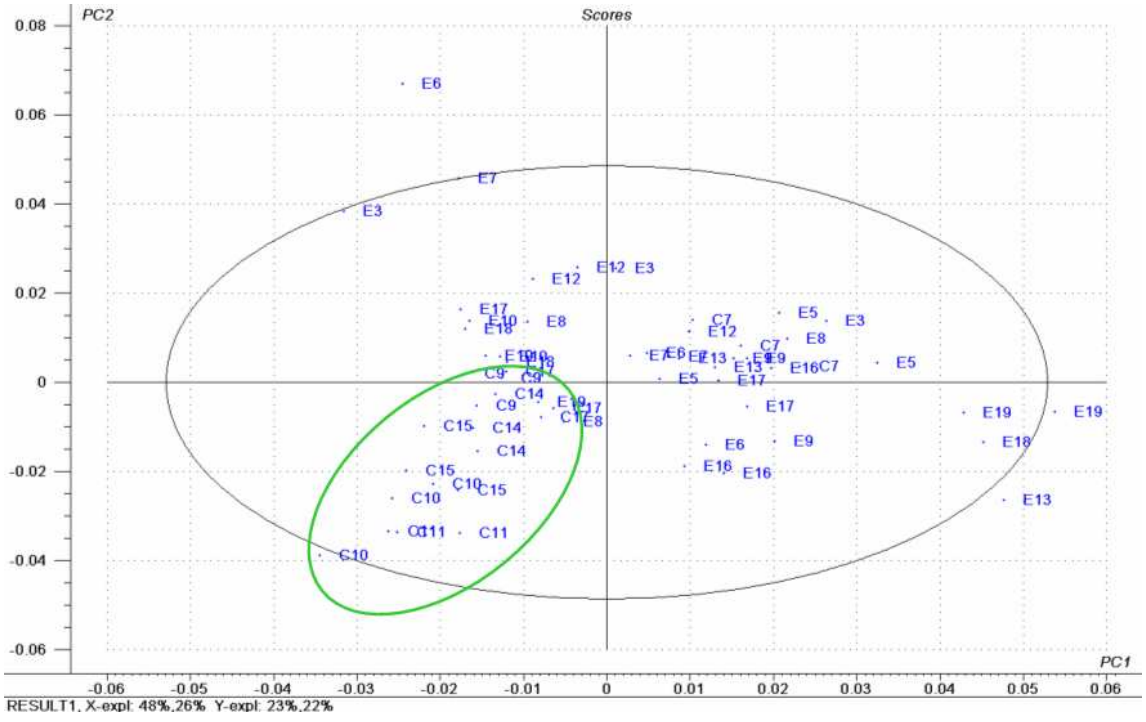


Fig. 38: PLS-DA scores plot (PC1 (48%) vs. PC2 (26%) of methanol extracts from Equine Grass Sickness (E) and control (C) samples.

PLS-DA was also carried out on chloroform extracts of *R. repens* samples from Equine Grass Sickness (E) and control sites (C). A five component model explained 83% of the variance within the x variables (spectra), with the first two components explaining 32%. 53% of this variation could be explained by the y variable (0 or 1). The scores plot of PC1/2 (Fig. 38) showed a cluster of all control sites with the exception of C7 and C17.

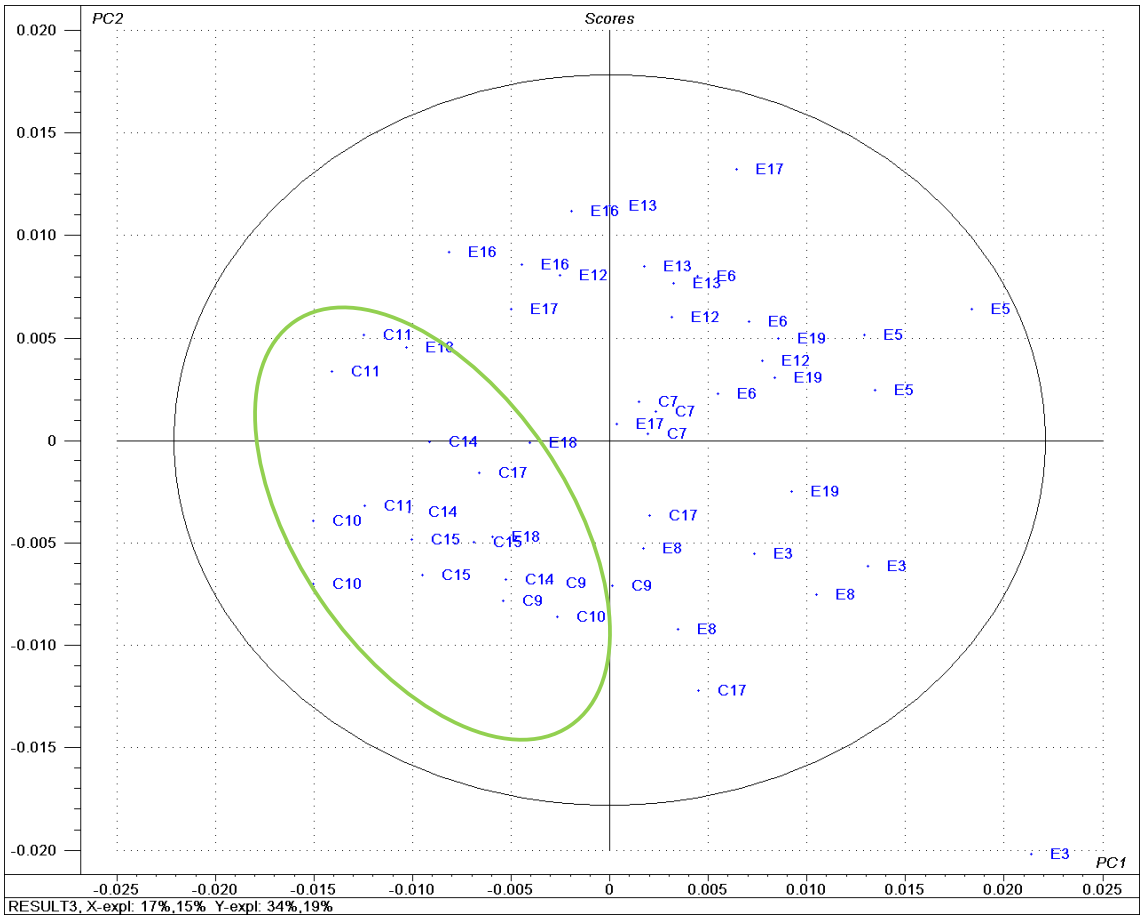


Fig. 39: PLS-DA scores plot (PC1 (17%) vs. PC2 (15%) of chloroform extracts from Equine Grass Sickness (E) and control (C) samples.

The loadings plot for PC1 (Fig. 41) showed positive loadings corresponding to the resonance peaks of the Ranunculin aglycon. Therefore samples with negative PC1 scores (Fig. 40) have lower contents of Ranunculin aglycon. Most control samples (with the exception of C7) have low PC1 scores, so their Ranunculin aglycon concentrations are approximately lower.

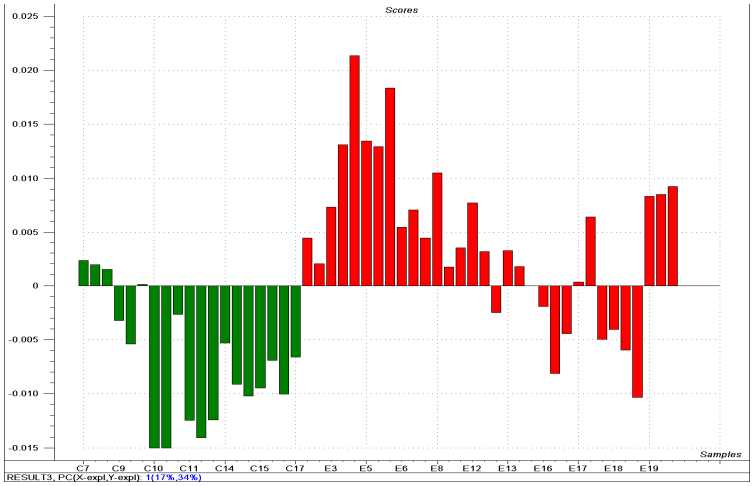


Fig. 40: PLS-DA scores plot (PC1 (17%)) of chloroform extracts from Equine Grass Sickness (red) and control samples (green)

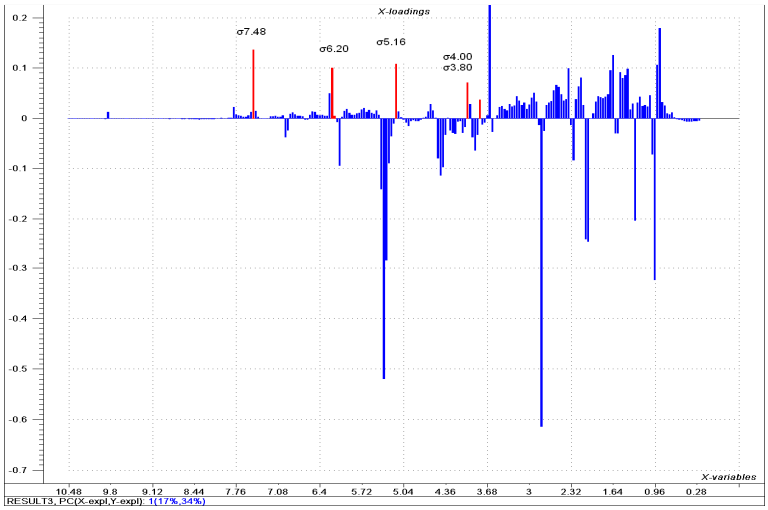


Fig. 41: PLS-DA loadings plot (PC1 (17%)) of chloroform extracts. Resonance peaks for Ranunculin aglycon (red).

3.3. PLS (partial least squares)

3.3.1. Number of EGS cases

The previous experiment was repeated with the y variable defined as the number of EGS cases that occurred at each site (see Table 3: Equine Grass Sickness sample data). A five component model explained 87% of the variance within the x variables (spectra), with the first two components explaining 58%. 41% of this variation could be explained by the y variable (number of EGS cases). The scores plot of PC1/2 showed a cluster of control sites, with the exception of sample C7. This result is similar to the results of PCA and PLS-DA analysis, but the classification is even more evident.

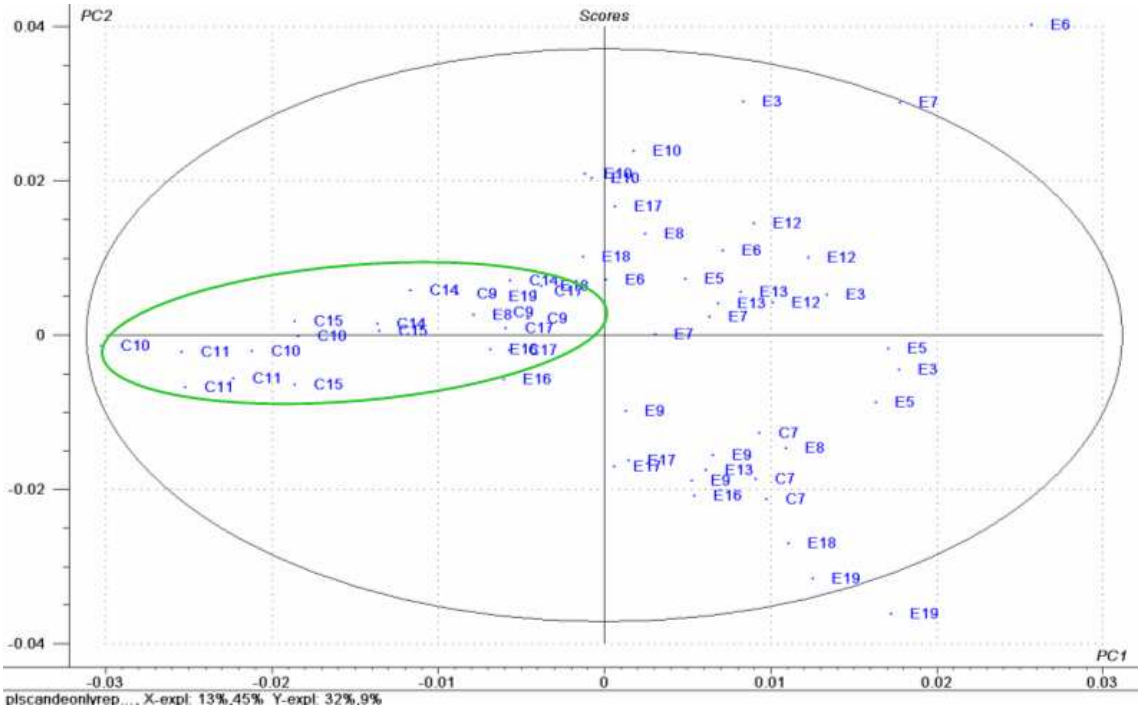


Fig. 42: PLS scores plot (PC1 (13%) vs. PC2 (45%)) of methanol extracts from Equine Grass Sickness (E) and control (C) samples.

The same experiment was carried out on the chloroform extracts. A five component model explained 84% of the variance within the x variables (spectra), with the first two components explaining 50%. 62% of this variation could be explained by the y variables (number of EGS cases). The scores plot shows clustering of control samples (with the exception of C7) mainly in the left lower half of the plot.

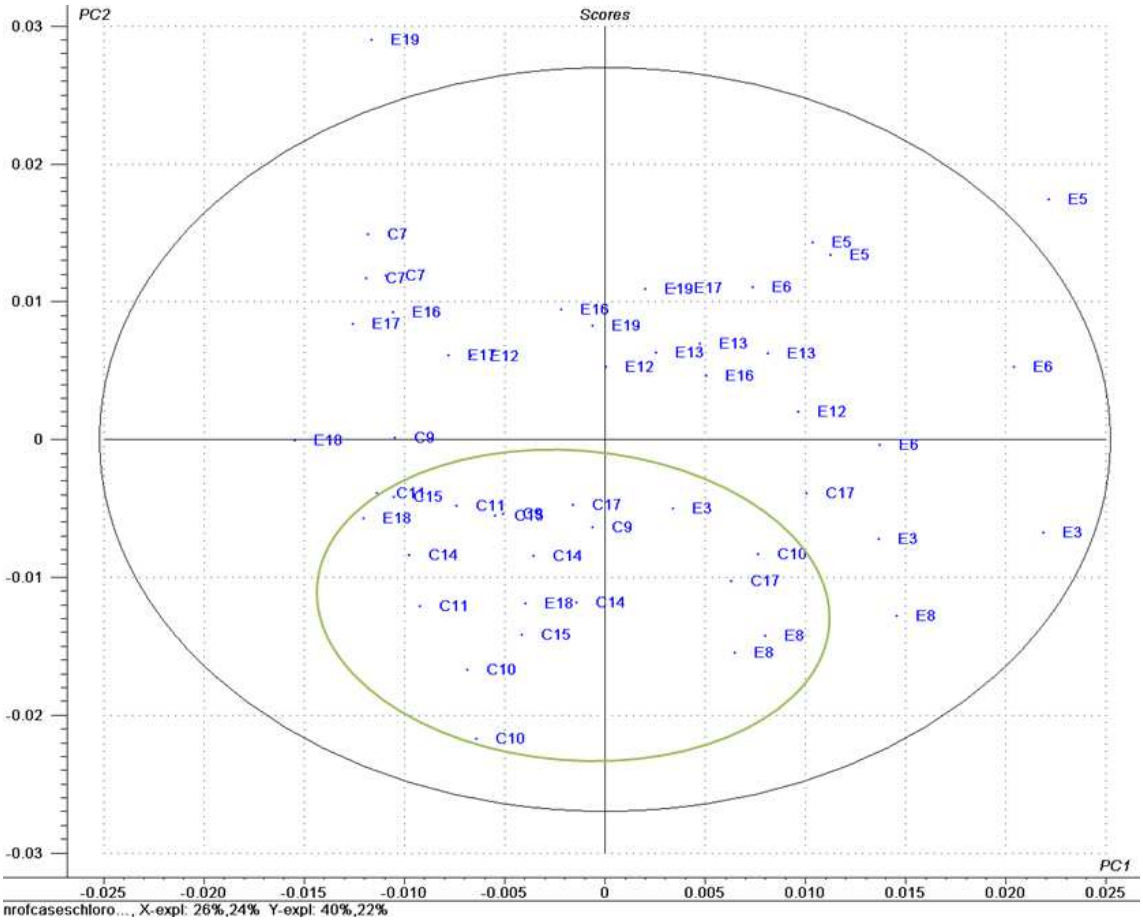


Fig. 43: PLS scores plot (PC1 (26% vs. PC2 (24%)) of chloroform extracts from Equine Grass Sickness (E) and control (C) samples.

The loadings plot for PC1 (Fig. 45) showed positive loadings corresponding to the resonance peaks of the Ranunculin aglycon. Therefore samples with negative PC1 scores (Fig. 44) have lower contents of Ranunculin aglycon. Most control samples (with the exception of C17) have low PC1 scores, so their Ranunculin aglycon concentrations are lower.

On the other hand there are also some EGS samples (E16, E17and E18) with low PC1 scores.

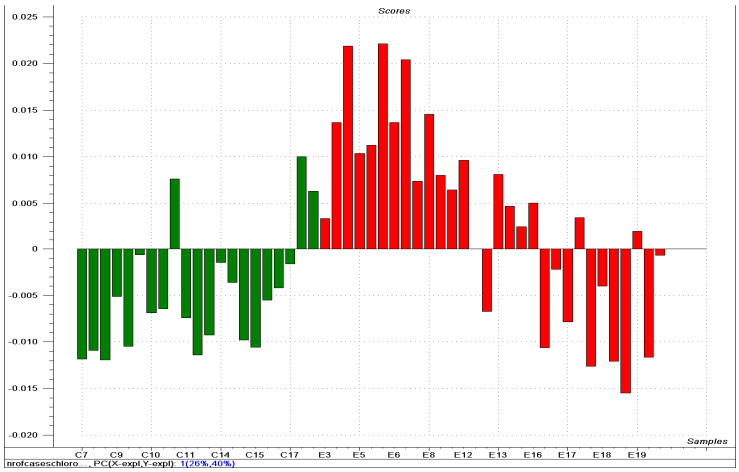


Fig. 44: PLS scores plot (PC1 (26%)) of chloroform extracts from Equine Grass Sickness (red) and control samples (green).

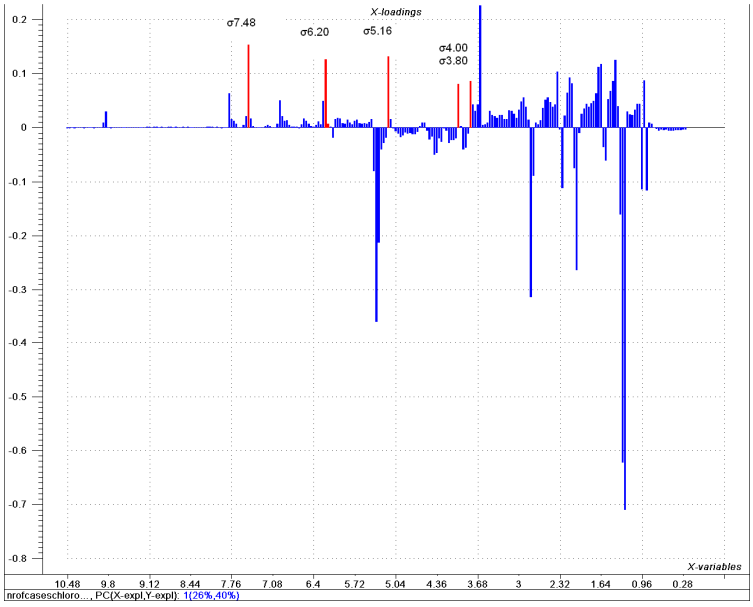


Fig. 45: PLS loadings plot (PC1 (26%)) of chloroform extracts. Resonance peaks for Ranunculin aglycon (red).

3.3.2. Soil analysis

For this experiment the y variable was defined as the level of properties of soil samples that had been collected at the same sites by Dr. Sarah Edwards (see Table 8 and Table 9 in the appendix). Nitrate levels are elevated in most samples from EGS sites. The more of the variation in the spectra can be explained by the variation in the soil property levels, the higher the possibility that there might be a relation. As can be seen in Fig. 46, a high percentage of the variation in the methanol extracts can be explained by the levels of ammonium nitrogen, available iron and fluoride.

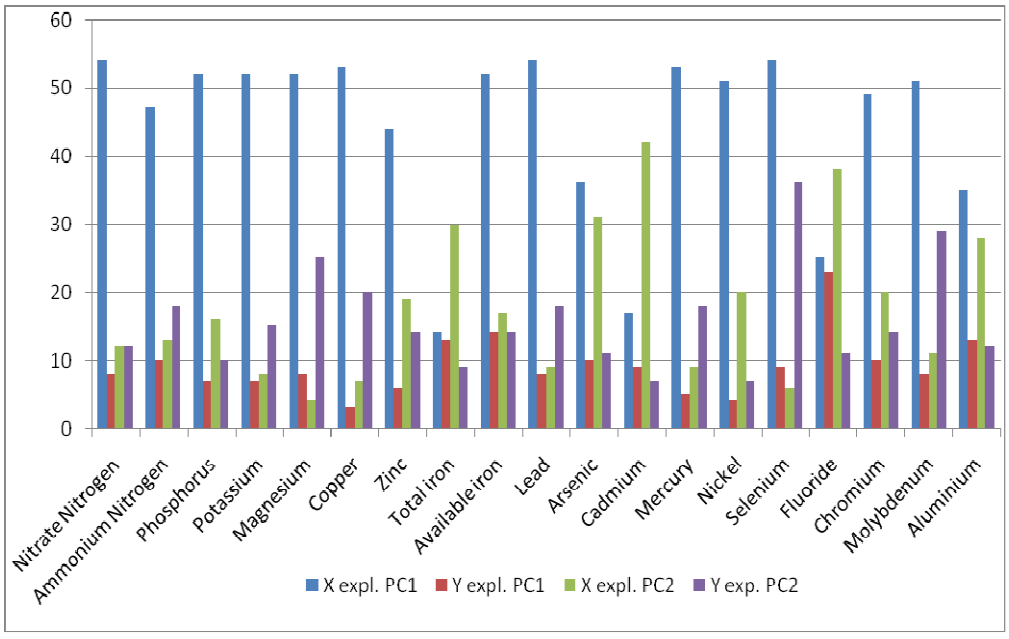


Fig. 46: Explained variances of X variables (spectra) and Y variables (soil properties) in % for the methanol extracts.

Fig. 47 showed that there might be a relationship between levels of nitrate nitrogen and total iron, chromium, fluoride, aluminium and the metabolic profiles of the chloroform extracts.

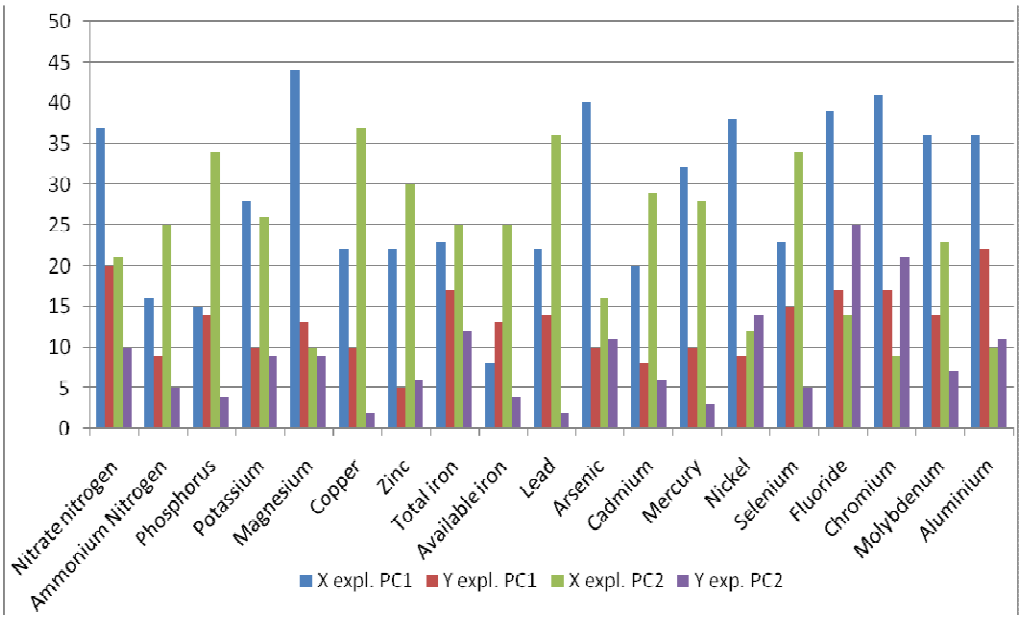


Fig. 47: Explained variances of X variables (spectra) and Y variables (soil properties) in % for the chloroform extracts.

All PLS scores plots (PC1/PC2) are displayed in the appendix (see page V).

The scores plots of nitrate nitrogen, cadmium and chromium show clustering between EGS and control samples of the methanol extracts, which might be a sign that these metals are involved in the aetiology of EGS. Looking at the chloroform extracts, there is clustering in the scores plot of available iron.

4. Discussion

4.1. Comparison of EGS and control samples

The aim of this study was to establish whether there is a significant difference between *Ranunculus* samples from EGS sites compared to those from control sites.

The results of the metabolomic analysis show that control samples (with exception of sample C7) group together and therefore have similar properties (see Fig. 33, Fig. 38 and Fig. 42). On the other hand, there is a lot of variation between the metabolic profiles of samples from EGS sites.

This might be a sign that there are environmental factors at EGS sites that increase metabolic stress for the plants that grow on these premises. This observation has also been made by McGorum et al.[54], who observed weak prooxidant activity of plant extracts collected at EGS pastures.

By means of partial least squares the variation in the extracts was correlated with the number of EGS cases that occurred at each site (Fig. 42). In the PLS analysis the grouping between control samples became even more evident. The EGS samples can approximately be divided into two groups that lie within close distance in the PLS scores. The first group consists of the samples E3, E5, E6, E7, E12 and E13, and the second group of samples E16, E17, E18, E19 and C7, which is an outlier of the control group.

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[54] McGorum et al. (2000)

A possible reason for this differentiation is that samples of the first group have been collected in 2007 (except E13, which has been collected in January 2008), and samples of the second group have been collected in 2008.

The time of freezing might have an impact on the metabolite composition of the plant material. Bai et al.[55] showed that Ranunculin hydrolyzes completely when stored in the freezer for 6 months. However, it is unclear how the time of freezing affects the entire metabolic profile.

Another possible explanation is that the climatic conditions during these two years were very different.

By observing scores plots (Fig. 36, Fig. 40 and Fig. 44) of the chloroform extracts it was possible to estimate that the majority of samples collected at equine grass sickness sites (E1, E2, E3, E5, E6, E7, E8, E10, E11, E12, and E18) contain elevated levels of Ranunculin aglycon. Even though there are also control samples with elevated Ranunculin aglycon levels (G2, C12, C13 and C14, see Fig. 16) these results support the hypothesis that *Ranunculus* is involved in the aetiology of Equine Grass Sickness.

The results suggest that not only high Ranunculin content but the whole range of plant metabolites might be involved in the aetiology of EGS (Fig. 33, Fig. 38 and Fig. 42). Nevertheless interpretation of these results is complicated, because both environmental as well as genetic factors have an impact on metabolic profiles of plants.

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[55] Bai et al (1996)

4.2. Comparison of different species

Metabolomics proved to be a powerful tool for showing the differences in the metabolite composition of the three species *R. repens*, *R. acris* and *R. bulbosus* (Fig. 15, Fig. 16 and Fig. 17). All *R. acris* samples, with the exception of sample C1, have high Ranunculin aglycon concentrations. Most *R. repens* samples have lower PC2 scores, indicating they have a lower Ranunculin content, but there are also some with high PC2 scores (E3, E4, E5, E7, E8, E19, C9 and G2).

Looking at the whole metabolic profile, it is not possible to define certain metabolites as being typical for a certain *Ranunculus* species (Fig. 17). However, it can be said that the variation in Ranunculin aglycon concentration is not one of the main differences between the metabolic profiles of different Ranunculus species.

Furthermore the results clearly showed that the chemical composition of *R. acris* and *R. bulbosus* are more similar compared to *R. repens.*

4.3. Comparison of different seasons

EGS cases occur mainly in spring or summer. Therefore most samples from EGS sites have been collected between May and August. Only samples E12 (December) and E13 (January) have been collected in winter. Control samples were collected at all times of the year to find out if there are seasonal changes in the metabolic profile. The results show that samples collect in winter differ from samples collected in spring or summer (Fig. 24and Fig. 30). However these are only minor differences, since they can only be observed in higher principal components. Therefore the impact of the season of collection is smaller than expected.

Furthermore, the results showed that the concentrations of Ranunculin aglycon are generally lower in winter, and elevated in spring (Fig. 28 and Fig. 29).
Since EGS outbreaks most likely occur in spring, the fact that toxin levels are elevated in spring supports the hypothesis that Ranunculus is involved in EGS.

4.4. Limitations

4.4.1. Geographical location

The metabolomics results show that all control sites (with the exception of C7) group together in one cluster (Fig. 33, Fig. 38 andFig. 42). One possible explanation for this could be that all control samples (with the exception of sample C10 from Angus and C9 from Northamptonshire) have been collected within close geographical distance. Some of the EGS samples were collected in different parts of Great Britain and within greater geographical distance. Therefore it is likely that there is more variation between their metabolic profiles.

4.4.2. Composition of the plant material

The metabolic profile is also dependent on the composition of the plant material. Most of the samples had no or hardly any flowers, with the exception of sample E19, which consisted almost only of flowers. The results of the metabolic analysis show sample E19 as an outlier (Fig. 14, Fig. 33 and Fig. 36). The theory that Ranunculin is more concentrated in the reproductive organs, such as flowers, cannot be supported

because the ^{1}H-NMR spectra showed that E19 had very low levels of Ranunculin aglycon.

Furthermore it should make a difference if the plant material contains a majority of stems or leaves. Variations in the composition of the plant material should be avoided, but plant material is always inhomogenous.

In order to minimize these variations and to ensure the reproducibility of the method, all samples have been extracted three times. Even though the extraction procedure was the same for all samples, the metabolomics results show differences for some samples within these repeats. With the exception of E3, E6, E18 and G8, the method showed good reproducibility for the three repeats (Fig. 14).

4.4.3. Time of freezing

Ranunculin and Protoanemonin are unstable compounds. The longer the samples are frozen the higher the levels of Ranunculin aglycon will be[56]. Samples that have been collected a long time ago (E1, E2, E3 and E5) have high concentrations of Ranunculin aglycon (Fig. 19).

4.4.4. Amount of Ranunculus

Although these results support the hypothesis that *Ranunculus* could be involved in the aetiology of equine grass sickness there are certain factors that cannot be

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[56] Bai et al. (1996)

included in a phytochemical analysis. First, the amount of *Ranunculus* that grows at the pastures. There might be premises on which *Ranunculus* with a high concentration of toxins is growing, but the prevalence for these plants is very low. On the other hand, there could be cases at farms where Ranunculus has very low levels of toxins, but the plants grow more frequently, so that a horse eats larger amounts of *Ranunculus.* Furthermore we do not know the amount of *Ranunculus* the horse actually eats. As mentioned before, horses generally avoid eating *Ranunculus*, but if other food is rare or if they develop a taste for it, it is possible.

4.5. Conclusion

The results of these studies support the hypothesis that *Ranunculus* is involved in the aetiology of Equine Grass Sickness.

By using principal component analysis it was possible to show a clear differentiation between samples from EGS sites and samples from control sites. Regression analysis results indicated that there is a relation between the variation of the chemical composition of the plants and the number of EGS cases that occurred at each farm.

Since the chemical composition of plants is dependent on various factors, like climatic condition and geographical location, interpretation of these results is very complicated.

Consequently, the results of this analysis cannot be seen as a definite proof that there is a relationship between Ranunculus and Equine Grass Sickness. For drawing final conclusions on this matter, further studies, phytochemical as well as pharmacological, are required.

5. Abstract

Equine grass sickness (EGS), or equine dysautonomia, is a polyneuropathy affecting the central, peripheral and enteric nervous systems of grazing horses. EGS occurs most frequently within Great Britain, although it is also recognized in regions of mainland Europe. It affects predominantly young horses with access to pasture in springtime. Although EGS has been recognized for nearly 100 years the cause has not been definitively determined. Recent studies showed that intoxication with *Clostridium botulinum* type C is involved in EGS, but it is very likely that EGS has a multifactorial aetiology.

The aim of this study was find out if there is a relation between phytochemical data of *Ranunculus* samples and EGS. To test this hypothesis a phytochemical analysis was carried out on *Ranunculus* samples from 12 sites where there have been EGS outbreaks as well as samples from 9 control sites where EGS has not occurred.

Fresh *Ranunculus* contains Ranunculin, a glycoside that is enzymatically hydrolyzed to produce Protoanemonin, a toxic metabolite that can cause blistering and gastrointestinal irritation. Therefore buttercups might be able to evoke lesions in the gastrointestinal tracts of the horses. This may allow the bacterium *Clostridium botulinum* to enter the bloodstream.

For the phytochemical analysis all *Ranunculus* samples were frozen, freeze-dried and extracted with methanol in a triplicate. A metabolic profiling carried out by means of ^{1}H-NMR spectroscopy and multivariate data analysis was applied to the crude methanol extracts, as well as their chloroform fractions. NMR spectra were compared with the statistical methods PCA (principal component analysis) as well as PLS-DA (partial least squares - discriminant analysis) and PLS (partial least squares).

Metabolomics proved to be a powerful technique not only to show a significant difference between samples from EGS sites and control sites, but it also showed the differences between different *Ranunculus* species and different metabolite profiles depending on what season the samples had been collected.

Another aim was to show if soil properties have an impact on the metabolic range of the *Ranunculus* samples. The results indicate that high levels of iron, nitrate or chromium might have an impact on the variation in the metabolite composition of the extracts.

6. Zusammenfassung

Equine grass sickness (EGS) oder Graskrankheit ist eine Polyneuropathie, die das zentrale, periphere und enterale Nervensystem von grasenden Pferden betrifft. EGS tritt häufig in Großbritannien auf, es kommt aber auch auf dem europäischen Festland vor. Die Krankheit befällt hauptsächlich junge Pferde mit Zugang zu Weiden im Frühling. Obwohl EGS seit über 100 Jahren bekannt ist, ist die Ursache bis heute ungeklärt. Neuere Studien deuten darauf hin dass Intoxikation mit *Clostridium butulinum* type C involviert sein könnte. Es ist jedoch sehr wahrscheinlich dass EGS eine multifaktorielle Krankheitsursache hat.

Das Ziel dieser Arbeit war herauszufinden ob es einen Zusammenhang zwischen phytochemischen Daten von *Ranunculus* Proben und EGS gibt. Um diese Hypothese zu beweisen wurden *Ranunculus* Proben von 12 Standorten gesammelt auf denen EGS Fälle aufgetreten sind und von 9 Standorten auf denen EGS noch nie vorgekommen ist.

Frische Hahnenfüße enthalten Ranunculin, ein Glykosid, aus welchem durch enzymatische Umwandlung der toxische Metabolit Protoanemonin gebildet wird. Durch die Reizwirkung von Protoanemonin können Läsionen im Gastrointestinaltrakt von Pferden entstehen. Das Bakterium *Clostridium botulinum* kann dadurch erleichtert in die Blutbahn gelangen.

Für die phytochemische Analyse wurden alle *Ranunculus* Proben tiefgekühlt, lyophilisiert und dreimal mit Methanol extrahiert. Sowohl von den Methanol-Extrakten, also auch von deren Chloroform-Fraktionen wurden ^{1}H-NMR Spektra aufgenommen. Die Spektra wurden mittels multivariater Datenanalyse verglichen. Die statistischen Methoden PCA (principal component analysis), PLS-DA (partial

least squares - discriminant analysis) und PLS (partial least squares) wurden verwendet.

Durch diese Methoden konnten signifikante Unterschiede zwischen Proben von EGS Standorten und Kontrollstandorten festgestellt werden. Außerdem war es möglich die Unterschiede in der Zusammensetzung unterschiedlicher *Ranunculus* Spezies zu zeigen und jahreszeitliche Schwankungen in der chemischen Zusammensetzung der Pflanzenproben nachzuweisen.

Ein weiteres Ziel war zu ermitteln, ob die Konzentration von bestimmten Bodenparametern sich auf die metabolische Zusammensetzung der gesammelten *Ranunculus* Proben auswirken könnten. Die Resultate zeigen, dass erhöhte Konzentrationen an Eisen, Nitrat oder Chrom einen Einfluss auf die Pflanzen haben könnten.

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8. Curriculum vitae

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Appendix

1. Sample data

Sample name	Time of freezing	Wet weight	Waters content	Weight of material packed into column	Weight of dry extract	Drug extract ratio
GM1/1	15 month	12.123	76.5%	2.784	512.25	5.4
GM1/2	15 month	4.810	75.8%	1.112	264.75	4.2
GM1/3	15 month	4.816	77.9%	0.990	274.79	3.6
GM2/2	11 month	5.843	81.8%	1.054	294.50	3.6
GM2/3	11 month	5.253	83.4%	0.828	225.24	3.7
GM2/4	11 month	3.681	86.4%	0.500	144.29	3.5
GM3/2	10 month	6.648	81.9%	1.204	199.82	6.0
GM3/3	10 month	2.748	79.0%	0.530	172.44	3.1
GM3/4	8 month	2.517	73.7%	0.663	178.73	3.7
GM4/1	8 month	15.436	69.3%	4.780	990.66	4.8
GM4/2	7 month	3.230	82.8%	0.532	119.58	4.4
GM4/3	7 month	2.423	80.6%	0.444	115.00	3.9
GM5/1	7 month	16.293	74.0%	4.216	810.69	5.2
GM5/2	7 month	2.554	77.5%	0.606	170.74	3.5
GM5/3	7 month	2.827	80.3%	0.593	145.43	4.1
GM6/1	6 month	2.532	77.1%	0.554	140.19	4.0
GM6/2	6 month	2.577	82.9%	0.365	101.87	3.6
GM6/3	6 month	2.862	81.4%	0.486	117.36	4.1
GM7/1	2 month	3.057	80.2%	0.418	104.10	4.0
GM7/2	2 month	4.223	80.2%	0.671	135.77	4.9
GM7/3	2 month	3.238	80.9%	0.454	131.46	3.5
GM8/1	1 month	4.224	92.5%	0.379	61.91	6.1
GM8/2	1 month	4.379	93.0%	0.367	80.04	4.6
GM8/3	1 month	5.641	93.7%	0.410	62.72	6.5

Sample name	Time of freezing	Wet weight	Water content	Weight of material packed into column	Weight of dry extract	Drug extract ratio
GM9/1	3 weeks	2.845	83.2%	0.415	89.63	4.6
GM9/2	3 weeks	4.248	77.6%	0.896	154.85	5.8
GM9/3	3 weeks	3.564	73.9%	0.873	98.57	8.9
GM10/1	2 weeks	3.725	85.0%	0.548	138.69	4.0
GM10/2	2 weeks	4.522	84.8%	0.736	143.45	5.1
GM10/3	2 weeks	3.666	81.9%	0.654	119.33	5.5
C1/1	15 month	2.300	58.0%	0.951	284.33	3.3
C1/4	14 month	2.842	65.8%	0.985	253.87	3.9
C1/5	11 month	2.883	73.8%	0.756	166.51	4.5
C7/1	8 month	3.160	75.2%	0.828	233.30	3.5
C7/2	8 month	2.680	57.8%	1.197	316.81	3.8
C7/3	8 month	3.318	57.5%	1.389	381.43	3.6
C8/1	8 month	2.638	26.5%	1.932	533.44	3.6
C8/2	8 month	2.796	55.8%	1.189	267.18	4.5
C8/3	8 month	2.871	62.2%	1.038	267.44	3.9
C9/1	7 month	2.790	76.7%	0.772	212.63	3.6
C9/2	7 month	2.941	83.5%	0.549	158.45	3.5
C9/3	7 month	3.843	81.5%	0.797	205.01	3.9
C10/1	7 month	2.560	78.8%	0.485	143.81	3.4
C10/2	7 month	2.607	79.6%	0.495	151.77	3.3
C10/3	7 month	2.570	77.3%	0.543	156.19	3.5
C11/1	7 month	2.843	69.3%	0.859	186.94	4.6
C11/2	7 month	2.735	70.0%	0.821	187.85	4.4
C11/3	7 month	2.804	73.7%	0.734	173.22	4.2
C12/1	7 month	1.644	53.6%	0.763	204.10	3.7
C12/2	7 month	1.646	65.9%	0.562	104.84	5.4
C12/3	7 month	1.522	60.8%	0.596	130.76	4.6

Sample name	Time of freezing	Wet weight	Water content	Weight of material packed into column	Weight of dry extract	Drug extract ratio
C13/1	7 month	1.878	33.3%	1.249	218.46	5.7
C13/2	7 month	2.397	59.7%	1.000	255.63	3.9
C13/3	7 month	2.428	69.0%	0.777	154.23	5.0
C14/1	7 month	2.191	60.3%	0.839	206.11	4.1
C14/2	7 month	2.191	73.5%	0.570	150.70	3.8
C14/3	7 month	3.376	74.4%	0.855	227.45	3.8
C15/1	7 month	2.582	74.1%	0.677	199.24	3.4
C15/2	7 month	2.564	77.1%	0.623	160.33	3.9
C15/3	7 month	2.986	78.1%	0.679	171.99	3.9
C16/1	7 month	1.886	31.3%	1.231	243.23	5.1
C16/2	7 month	1.929	44.5%	1.037	181.02	5.7
C16/3	7 month	2.257	55.9%	0.970	209.43	4.6
C17/1	6 month	2.361	61.1%	0.934	237.57	3.9
C17/2	6 month	2.913	75.6%	0.765	224.31	3.4
C17/3	6 month	2.895	79.5%	0.610	163.32	3.7
EGS1/1	23 month	3.125	75.9%	0.784	167.17	4.7
EGS1/2	23 month	3.438	63.2%	1.298	474.76	2.7
EGS1/3	23 month	3.260	75.8%	0.817	200.05	4.1
EGS2/1	23 month	1.288	28.6%	0.874	197.29	4.4
EGS2/2	23 month	1.824	39.5%	1.049	269.57	3.9
EGS2/3	23 month	1.697	41.0%	0.957	174.50	5.5
EGS3/1	23 month	2.919	59.0%	1.175	354.27	3.3
EGS3/2	23 month	3.130	66.6%	1.029	360.37	2.9
EGS3/3	23 month	2.504	72.3%	0.689	181.85	3.8
EGS5/1	23 month	3.235	75.1%	0.871	158.02	5.5
EGS5/2	23 month	3.732	70.9%	1.115	240.86	4.6
EGS5/3	23 month	4.924	67.9%	1.182	174.93	6.8

Sample name	Time of freezing	Wet weight	Water content	Weight of material packed into column	Weight of dry extract	Drug extract ratio
EGS6/1	23 month	2.269	57.3%	0.952	252.66	3.8
EGS6/2	23 month	2.833	63.5%	1.046	176.12	5.9
EGS6/3	23 month	3.698	69.1%	1.130	253.54	4.5
EGS7/1	23 month	2.619	65.5%	0.888	375.46	2.4
EGS7/2	23 month	3.100	84.7%	0.469	122.08	3.8
EGS7/3	23 month	3.774	76.5%	0.885	280.92	3.2
EGS8/1	22 month	3.489	77.4%	0.815	245.82	3.3
EGS8/2	22 month	3.781	86.8%	0.519	144.99	3.6
EGS8/3	22 month	2.955	71.8%	0.866	239.60	3.6
EGS9/1	21 month	2.403	56.3%	1.024	198.54	5.2
EGS9/2	21 month	1.865	57.5%	0.806	108.27	7.4
EGS9/3	21 month	2.624	63.7%	0.952	122.94	7.7
EGS10/1	20 month	3.047	74.1%	0.863	289.06	3.0
EGS10/2	20 month	4.463	84.3%	0.713	245.48	2.9
EGS10/3	20 month	4.271	83.0%	0.735	256.81	2.9
EGS11/1	20 month	2.613	60.3%	1.025	122.63	8.4
EGS11/2	20 month	3.055	62.2%	1.127	98.73	11.4
EGS11/3	20 month	3.265	59.4%	1.314	147.68	8.9
EGS12/1	16 month	4.205	80.5%	0.795	235.75	3.4
EGS12/2	16 month	3.322	74.5%	0.855	272.75	3.1
EGS12/3	16 month	6.221	79.5%	1.251	299.84	4.2
EGS13/1	15 month	4.118	76.7%	0.924	213.91	4.3
EGS13/2	15 month	3.054	84.4%	0.460	109.57	4.2
EGS13/3	15 month	4.854	87.6%	0.627	161.54	3.9
EGS14/1	12 month	3.383	63.3%	1.257	272.18	4.6
EGS14/2	12 month	3.141	56.0%	1.458	389.72	3.7
EGS14/3	12 month	2.847	40.1%	1.716	397.40	4.3

Sample name	Time of freezing	Wet weight	Water content	Weight of material packed into column	Weight of dry extract	Drug extract ratio
EGS15/1	12 month	2.910	49.3%	1.461	379.84	3.8
EGS15/2	12 month	3.567	58.0%	1.466	393.73	3.7
EGS15/3	12 month	3.731	64.1%	1.330	379.49	3.5
EGS16/1	12 month	1.978	63.2%	0.729	183.56	4.0
EGS16/2	12 month	1.827	59.7%	0.739	158.94	4.6
EGS16/3	12 month	1.845	53.5%	0.731	172.91	4.2
EGS17/1	12 month	7.861	87.4%	0.991	243.89	4.1
EGS17/2	12 month	6.029	84.9%	0.864	177.63	4.9
EGS17/3	12 month	9.477	92,2%	0.742	171.15	4.3
EGS18/1	12 month	1.710	25,7%	1.235	385.89	3.2
EGS18/2	12 month	2.916	53,9%	1.345	403.03	3.3
EGS18/3	12 month	1.863	48,5%	0.994	254.31	3.9
EGS19/1	10 month	2.951	78,3%	0.556	114.72	4.8
EGS19/2	10 month	2.992	79,2%	0.583	151.52	3.8
EGS19/3	10 month	1.891	71,2%	0.508	147.22	3.5

2. Soil parameters

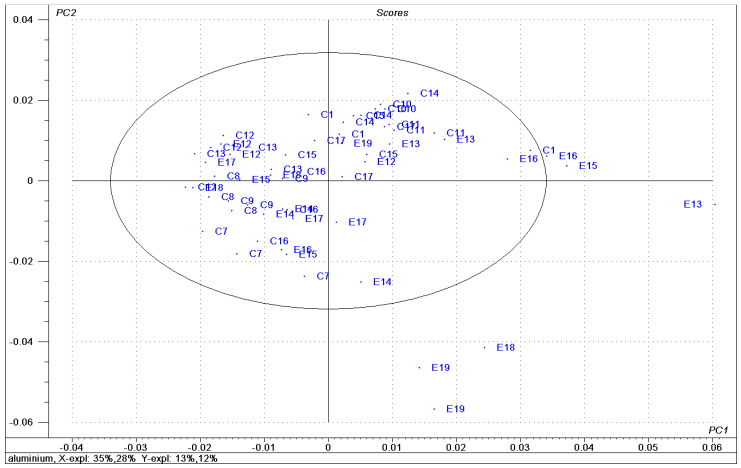
Table 8: Parameters of soil samples collected at control sites (C).

	C1	C7 C8	C9	C10	C11 C12	C13	C14 C16	C15	C17
Nitrate Nitrogen mg/kg	11.28	10.8	24.9	23.2	7.44	9.95	23.4	7.57	16.03
Ammonium Nitrogen mg/kg	12.76	18	9.64	7.09	13	3.78	4.34	4.71	21.7
Phosphorus mg/l	38.40	55.60	54.80	33.60	34.20	14.00	14.00	8.00	12.60
Potassium mg/l	535	224	227	248	410	126	266	114	201
Magnesium mg/l	318	147	76	126	103	111	104	100	97
Copper mg/kg	27.40	11.70	12.70	14.30	12.00	26.40	12.60	28.00	13.70
Zinc mg/kg	98.50	49.50	77.80	53.20	35.60	67.60	55.10	73.10	50.10
Total iron mg/kg	17688	18976	41395	18883	11435	18810	13617	26103	14409
Available iron mg/kg	319.7	518.10	140.20	181.50	335.00	248.30	347.1	144.00	178.00
Lead mg/kg	109.0	43.20	55.90	28.00	25.00	52.80	40.50	67.50	32.10
Arsenic mg/kg	10.6	9	49.2	9.9	6	11	5	14.3	5.7
Cadmium mg/kg	0,41	0.24	0.91	0.23	0.18	0.39	0.24	0.75	0.22
Mercury mg/kg	0.3	0.07	0.02	0.08	0.04	0.07	0.05	0.18	0.04
Nickel mg/kg	12.2	10.3	18.7	16.9	10	21.5	12.4	38.8	11.1
Selenium mg/kg	0.44	0,33	0.24	0.37	0.27	0.35	0.33	0.31	0.26
Fluoride mg/kg	31.3	21.4	17.6	25.2	12.6	11.8	11	13.9	23.1
Chromium mg/kg	28.3	15.2	39.4	25.7	16.3	24.4	18.7	18	30.6
Molybdenum mg/kg	1.9	1.3	1	0.6	1	0.9	0.6	1.2	0.9
Aluminium mg/kg	7884	5699	4437	10738	6350	10599	9524	9443	14037

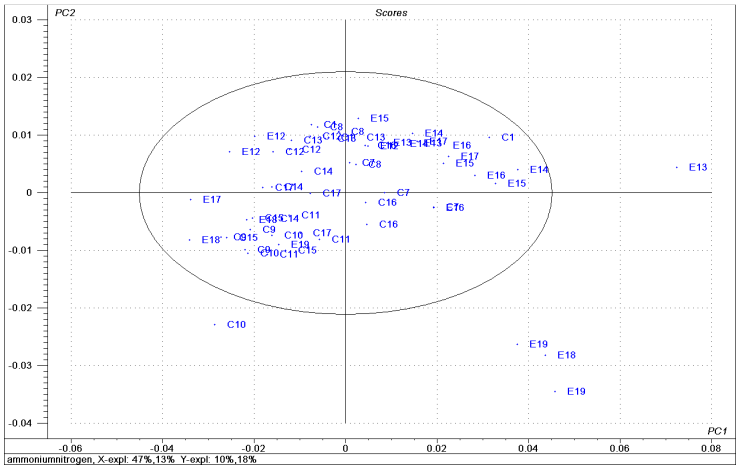
Table 9: Parameters of soil samples collected at Equine Grass Sickness sites (E).

	E1	E2	E3	E5 E6	E7	E8	E9	E10 E11	E12	E13	E14 E15	E17 E18	E19
Nitrate Nitrogen mg/kg	44.5	44.9	27.0	37.6	24.0	26.9	62.0	23.3	3.8	0.1	3.9	9.8	7.8
Ammonium Nitrogen mg/kg	18.1	69.4	23.9	7.99	0.97	1.66	3.02	1.45	11.4	26.2	17.5	5.62	2.28
Phosphorus mg/l	48.0		46.0	36.8	66.2	46.0	55.8	27.8	32.0	15.0	25.4	72.8	0.0
Potassium mg/l				127	495	261	431	134	61	38	101	215	0
Magnesium mg/l				70	203	124	87	191	95	82	35	99	0
Copper mg/kg	13.3	20.1	6.3	31.2	17.1	13.8	19.1	30.3	26.5	16.5	23.9	29.0	8.6
Zinc mg/kg	86.1	120.0	42.4	134.0	70.4	76.1	72.0	105.0	76.8	106.0	81.3	241.0	34.2
Total Fe mg/kg	21309	25176	8602	64672	17474	13800	13574	20331	15823	29703	22319	16489	12930
Available Fe mg/kg	568.0		325.0	214.0	334.8	426.3	253.9	376.7	496.0	311.0	146.3	171.6	0.00
Lead mg/kg	43.8	36.2	16.0	49.5	31.30	25.60	50.60	62.00	29.70	65.00	60.10	44.90	21.80
Arsenic mg/kg	10.8	10.6	4.5	29.5	10.3	6.3	7.5	12.7	5.7	11.3	10.8	21.3	7.7
Cadmium mg/kg	0.28	0.24	0.12	0.67	0.25	0.16	0.26	0.44	0.54	0.4	0.41	0.53	0.2
Mercury mg/kg	0.08	0.08	0.07	0.08	0.05	0.03	0.12	0.19	0.07	0.11	0.1	0.03	0.04
Nickel mg/kg	21	35.1	10.3	32.1	13.1	10	13.8	23.4	15.4	15.1	18	16.2	10.5
Selenium mg/kg	0.34	0.58	0.27	0.36	0.18	0.17	0.22	0.48	0.68	0.7	0.52	0.27	0.27
Fluoride mg/kg				86.9	32.7	13.2	18.8	26.5	31.1	30.8	11.8	13.4	12.7
Chromium mg/kg	37.4	60.2	18	57.5	24.6	23	18.8	41.4	24.4	32.2	19.7	29.8	12
Molybdenum mg/kg	1	1.8	0.7	0.2	0.6	0.4	0.7	0.5	2.8	1.3	1	1.2	0.4
Aluminium mg/kg									6067	13509	9574	4811	5242

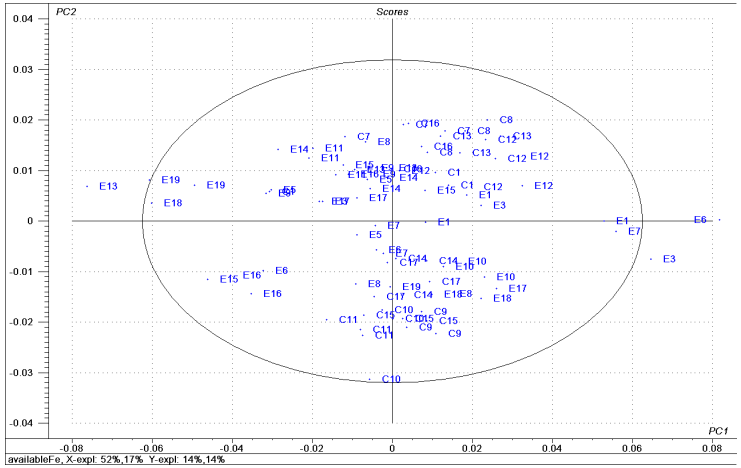
3. PLS results (PC 1 vs. PC2) of *R. repens* methanol extracts



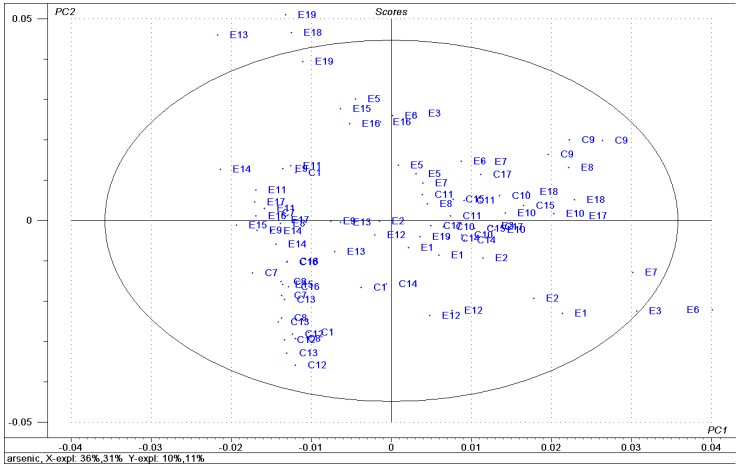
aluminium



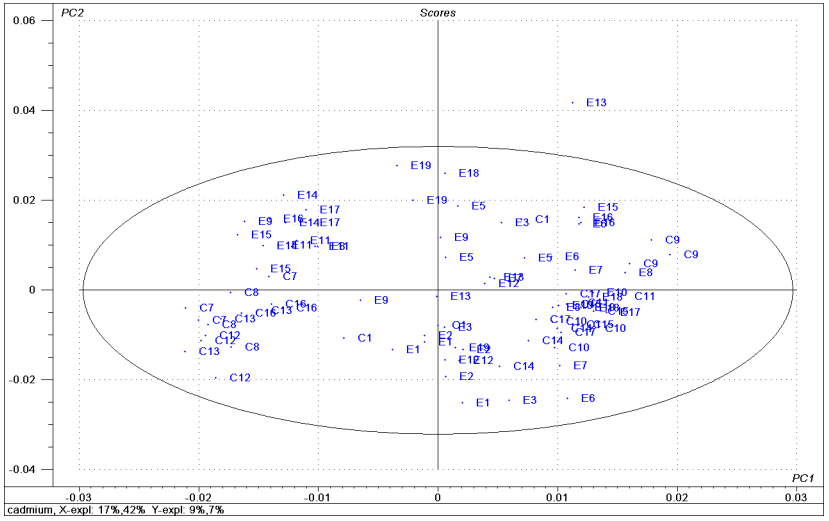
ammonium nitrogen



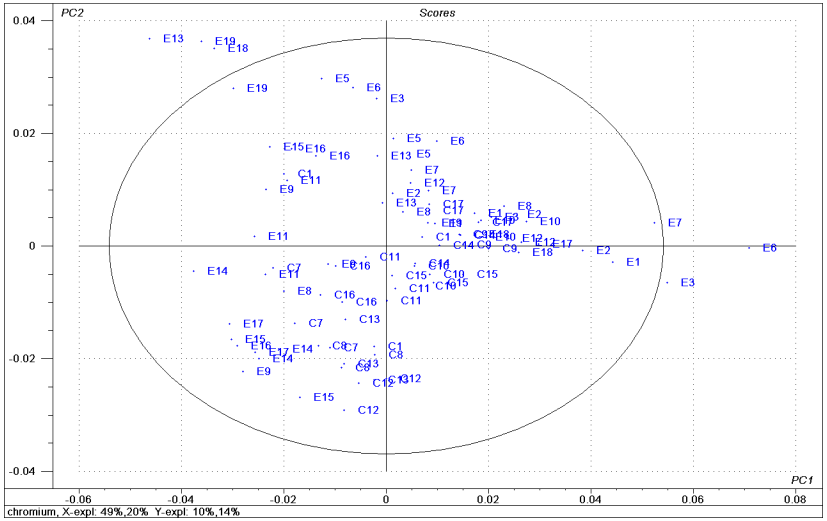
available iron



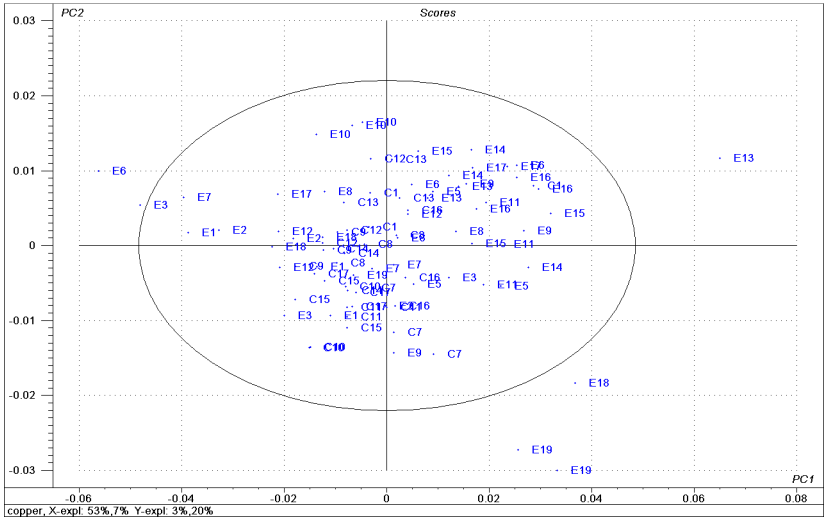
arsenic



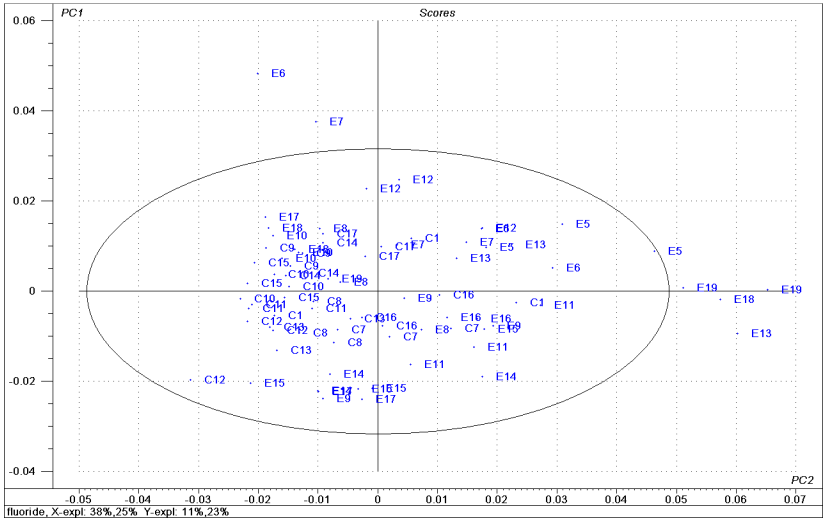
cadmium



chromium

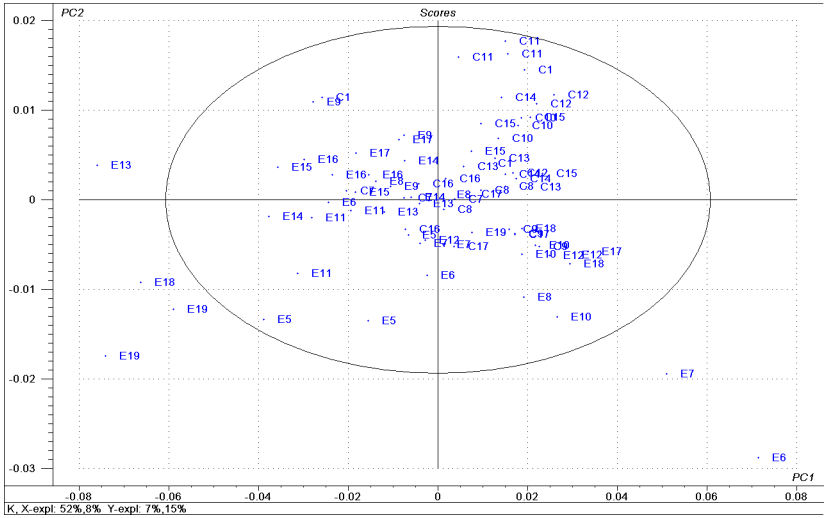


copper

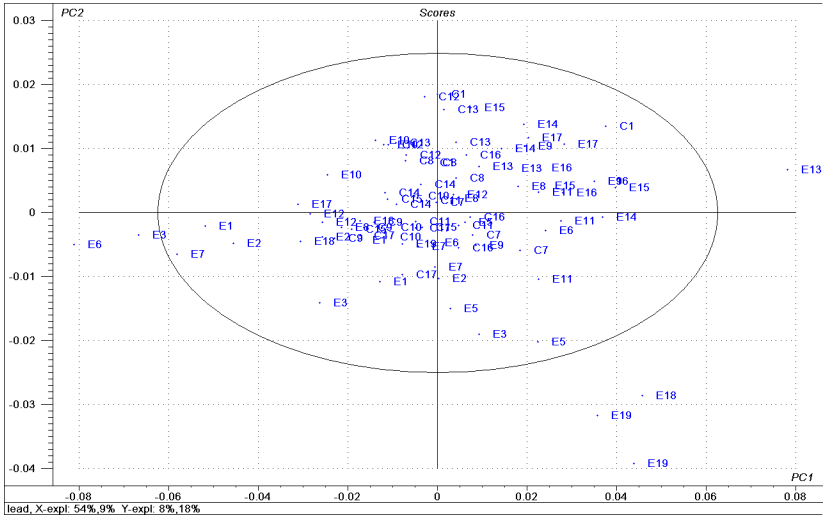


fluoride

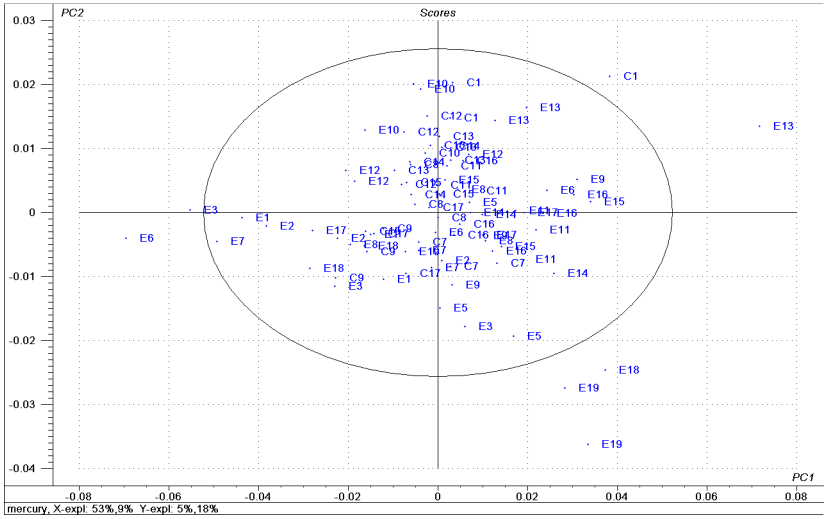
X



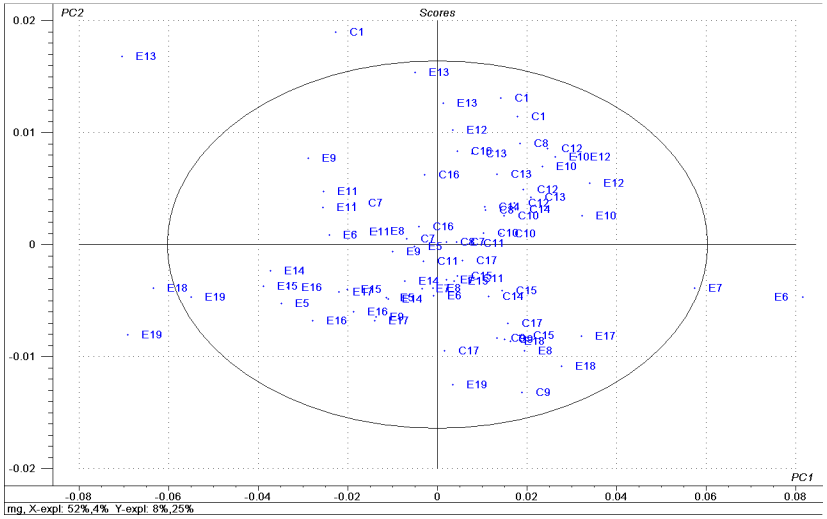
potassium



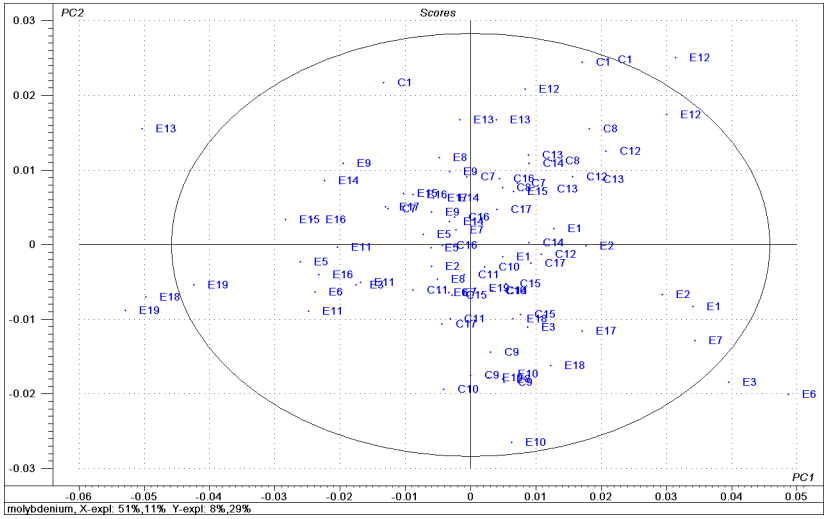
lead



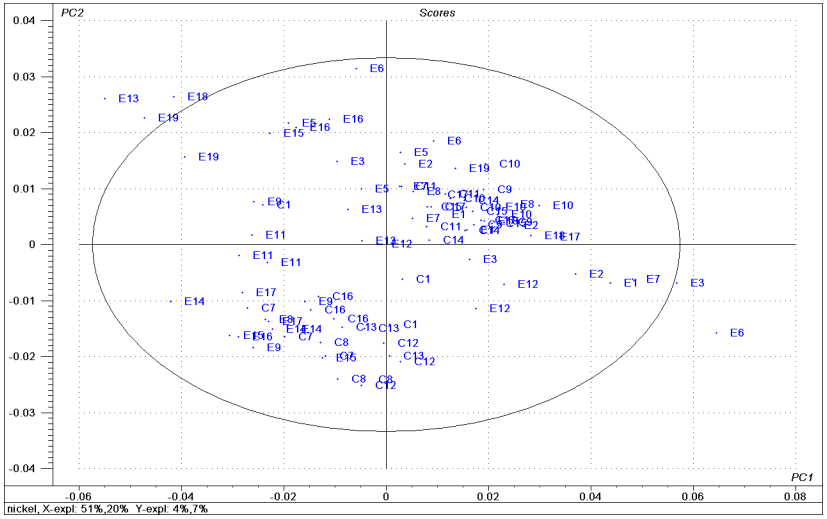
mercury



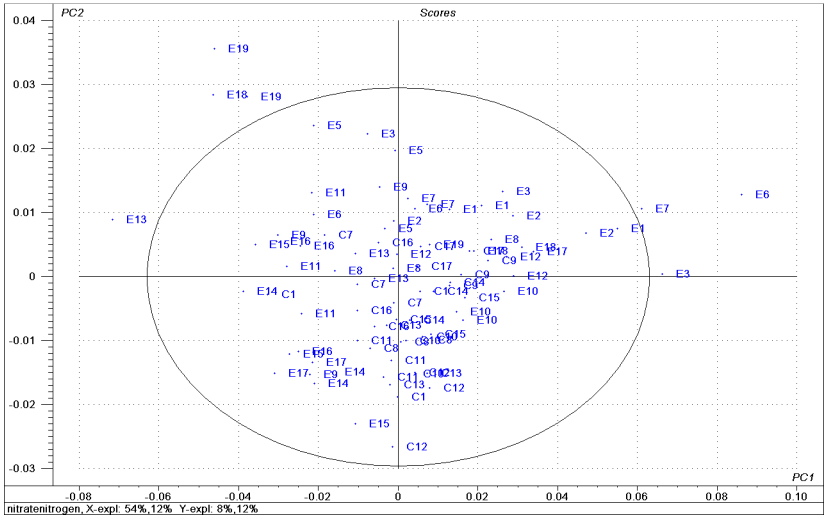
magnesium



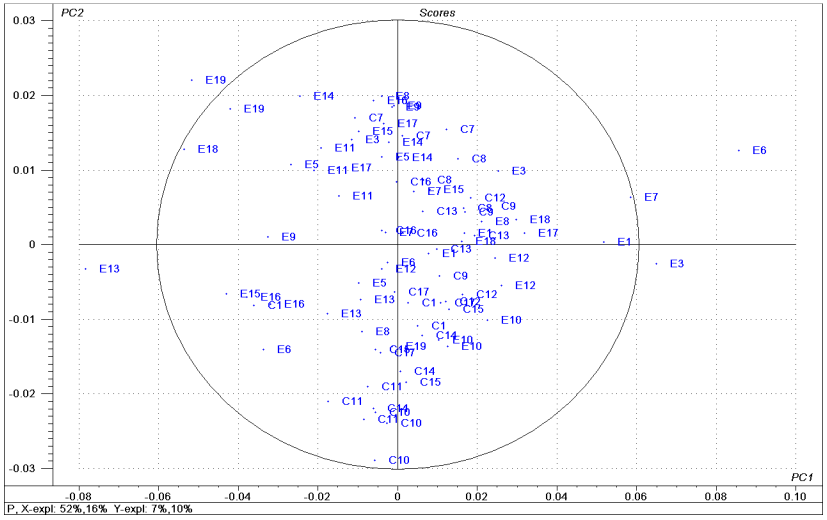
molybdenum



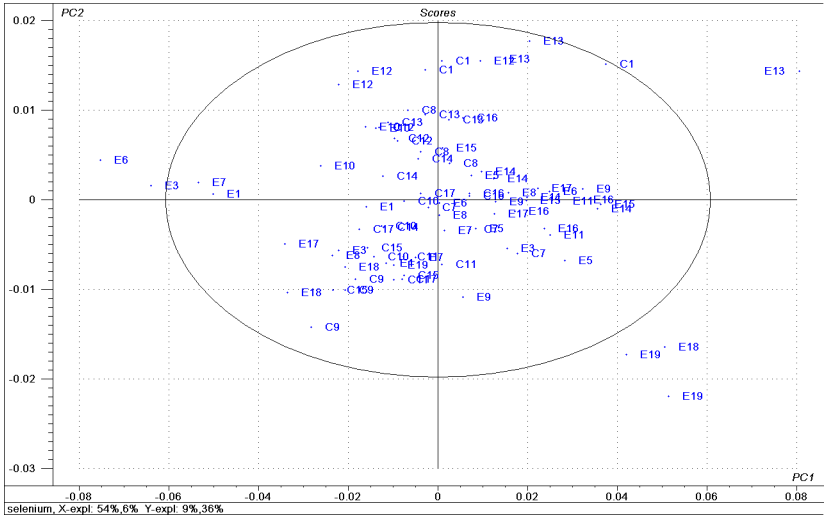
nickel



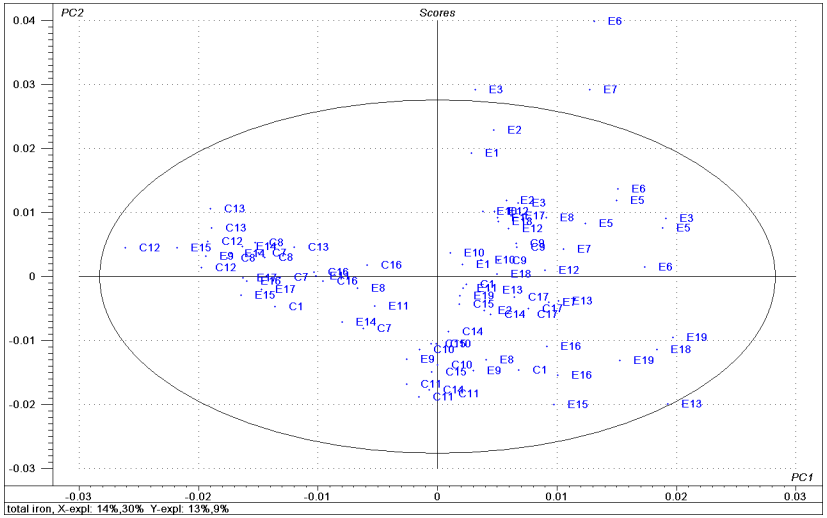
nitrate nitrogen



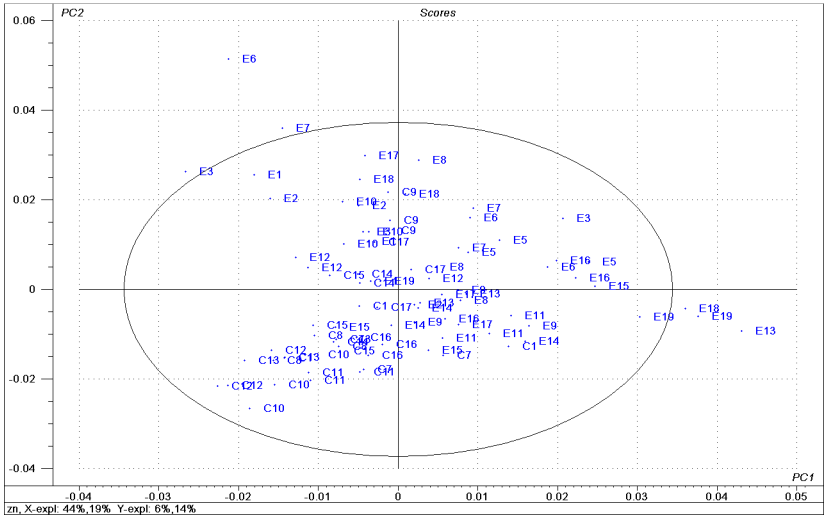
phosphorus



selenium

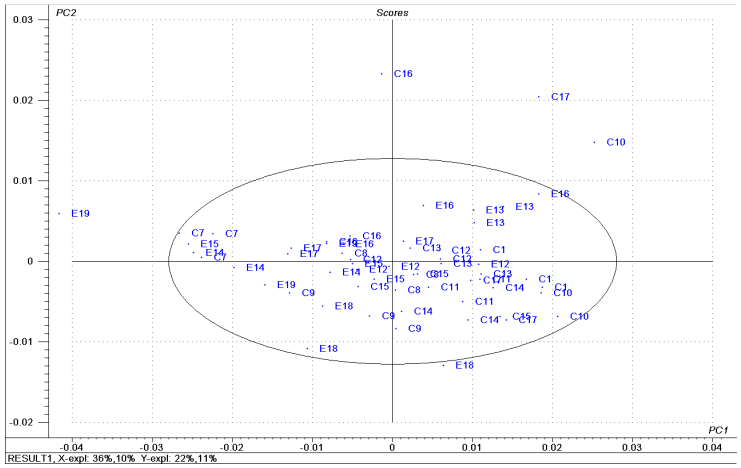


total iron

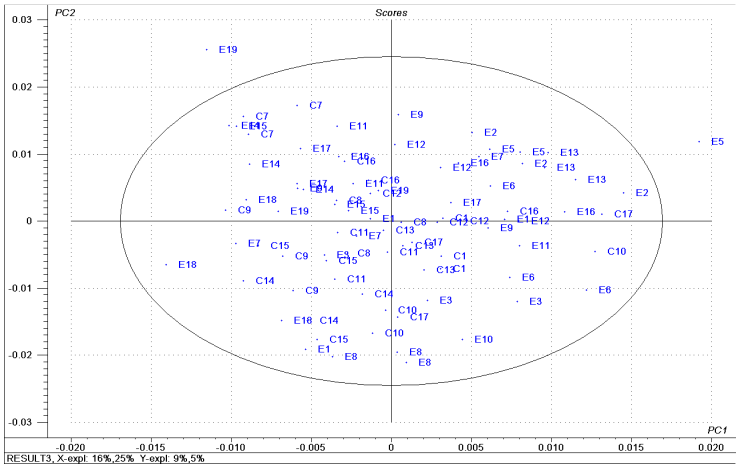


zinc

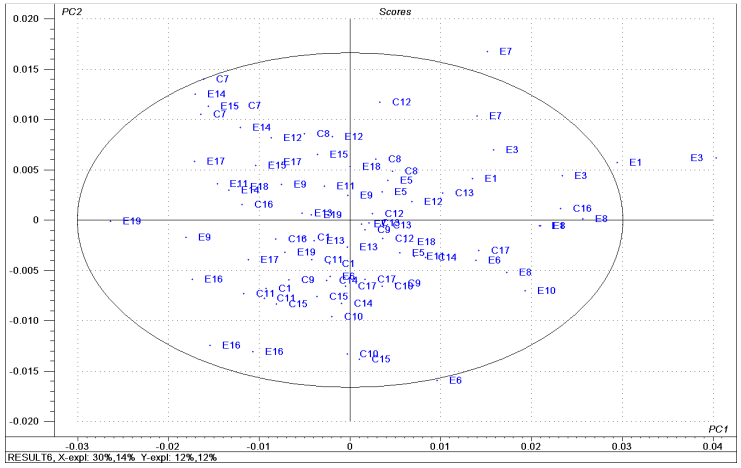
4. PLS results (PC 1 vs. PC2) of *R. repens* chloroform extracts



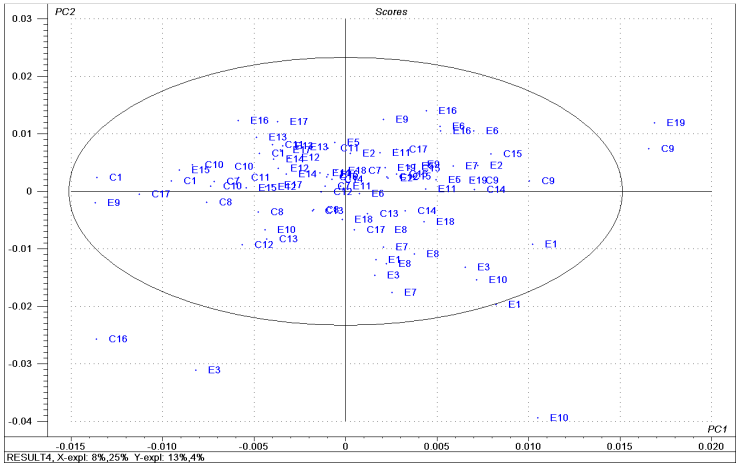
aluminium



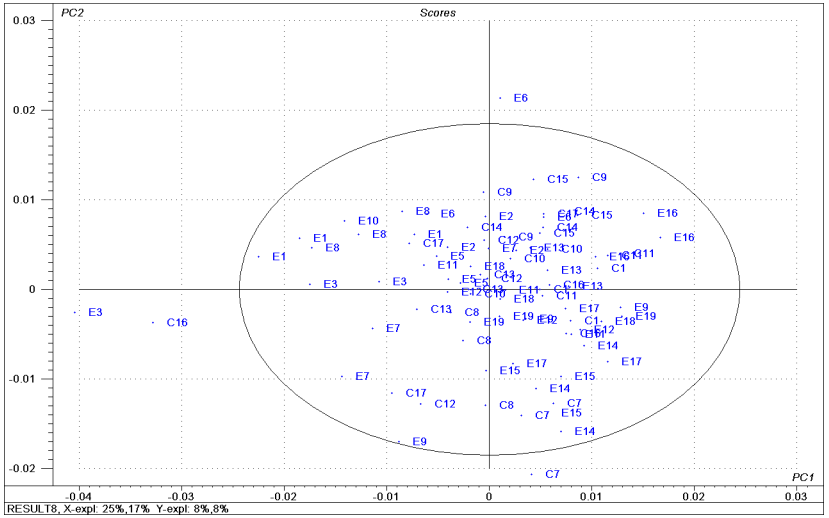
ammonium nitrogen



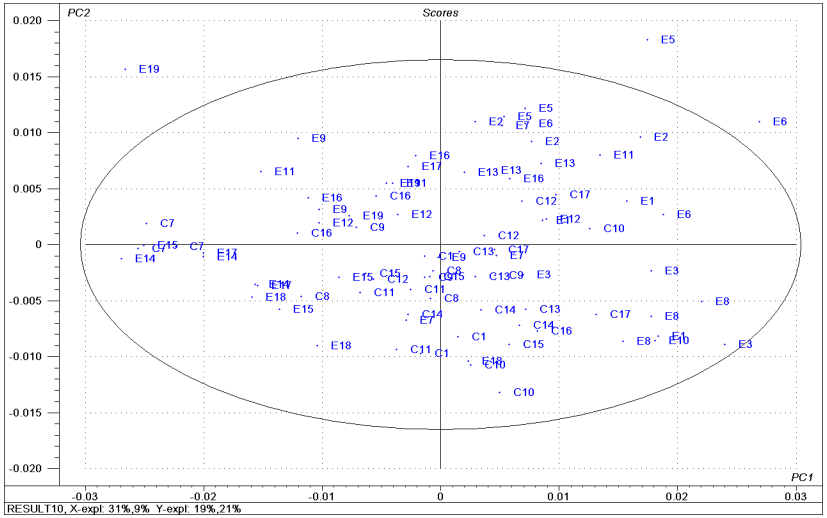
available iron



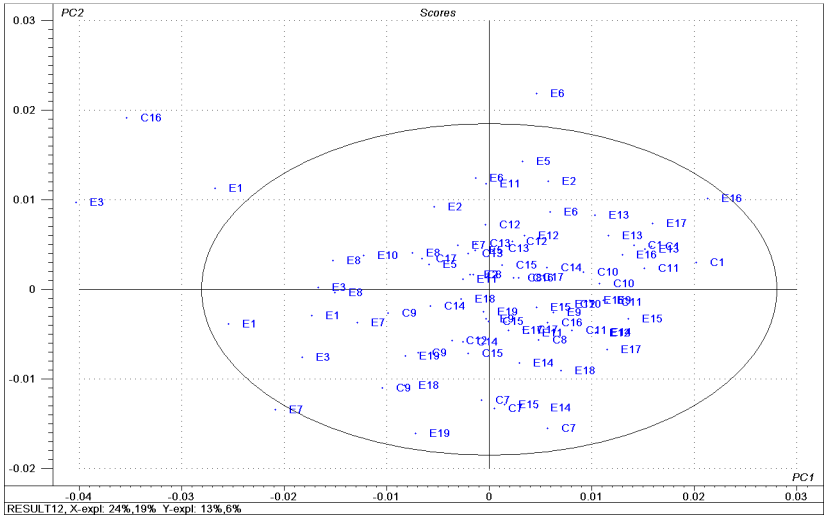
arsenic



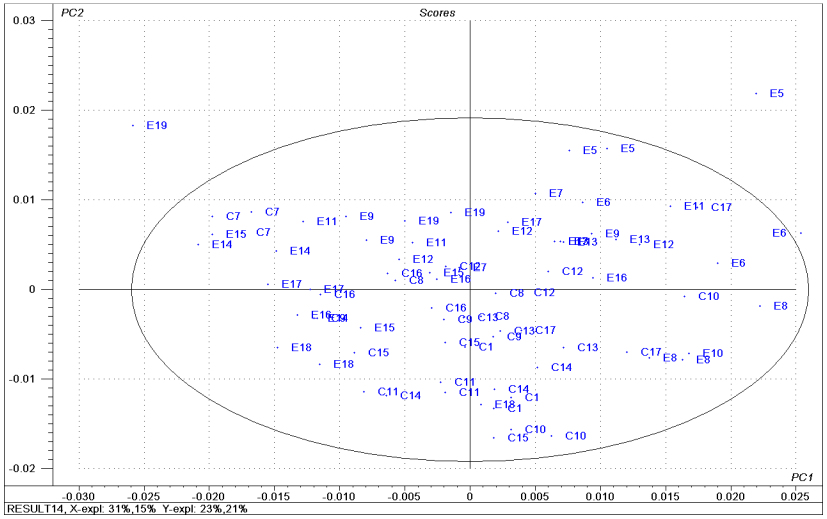
cadmium



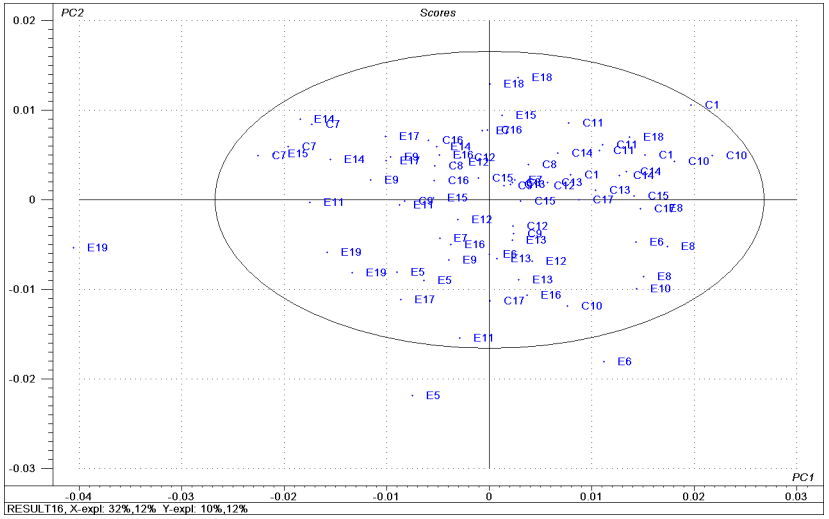
chromium



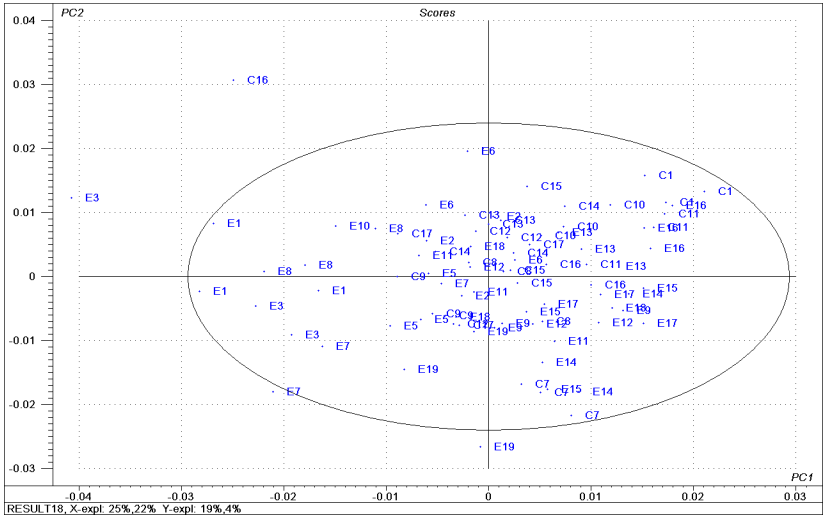
copper



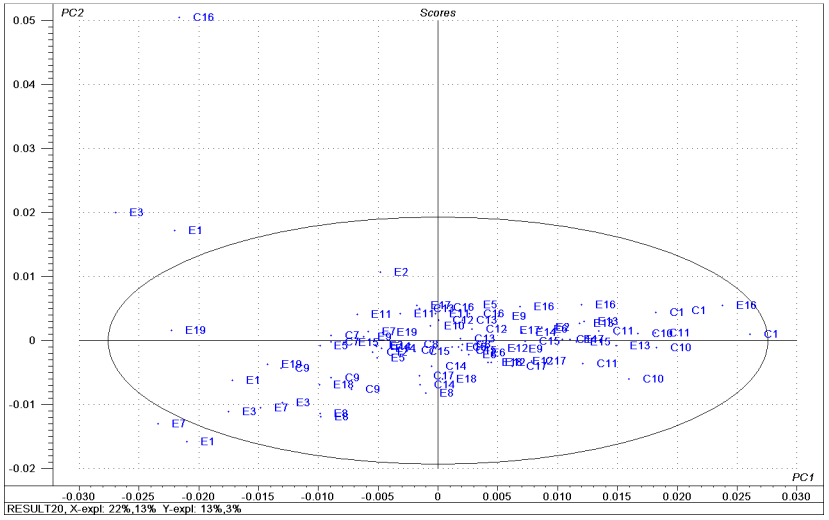
fluoride



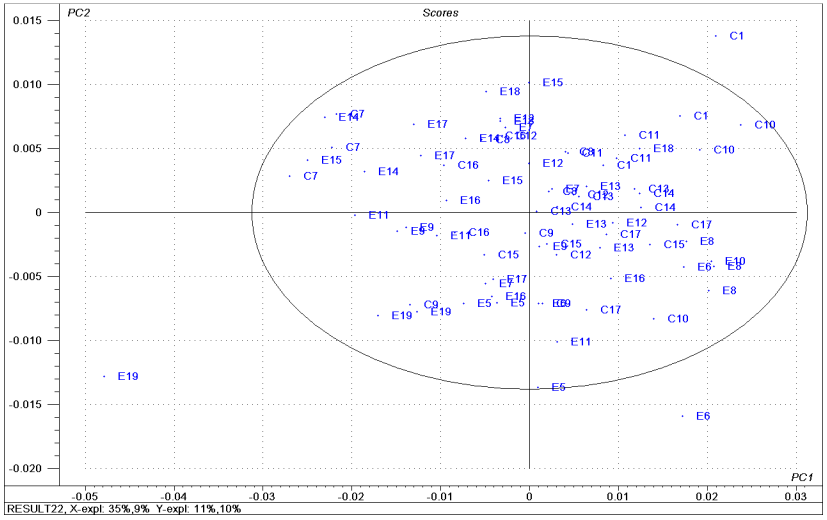
potassium



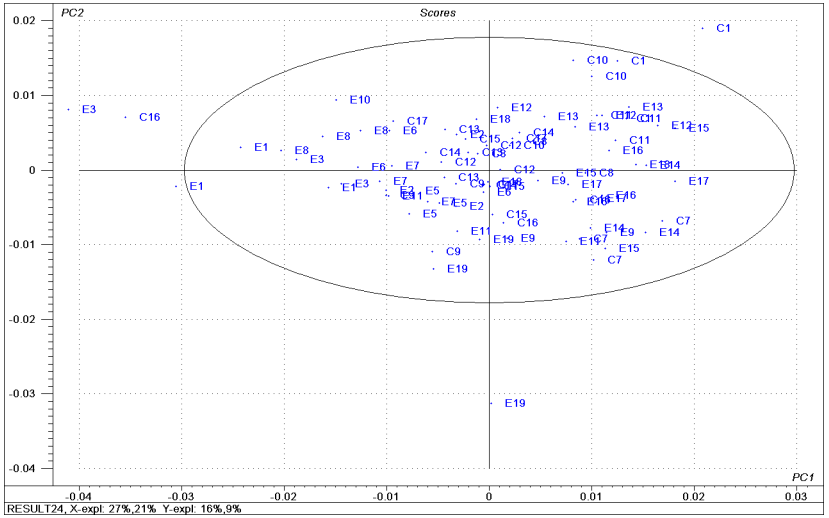
lead



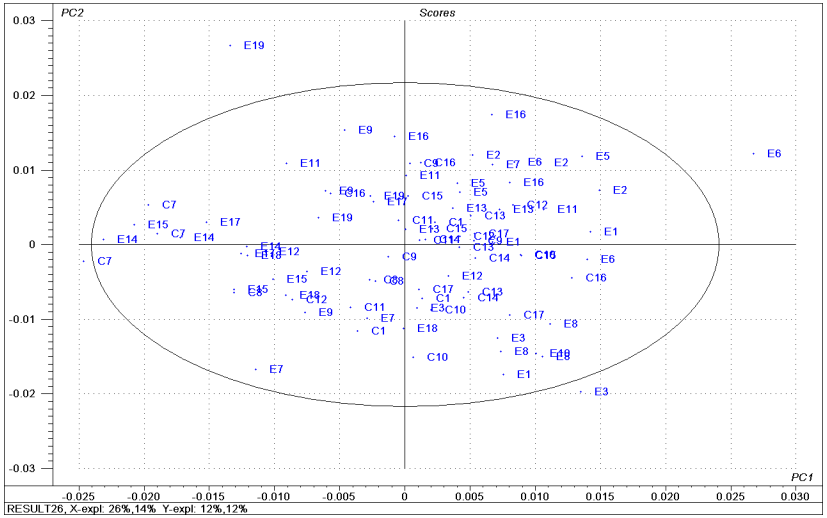
mercury



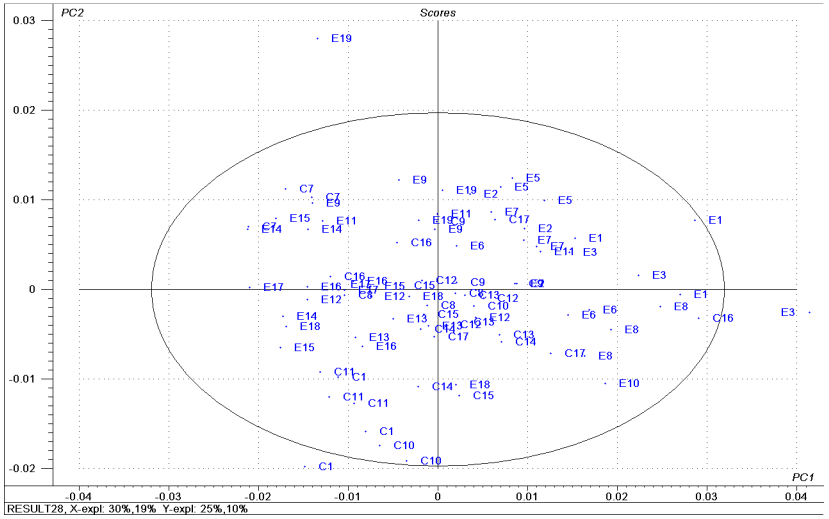
magnesium



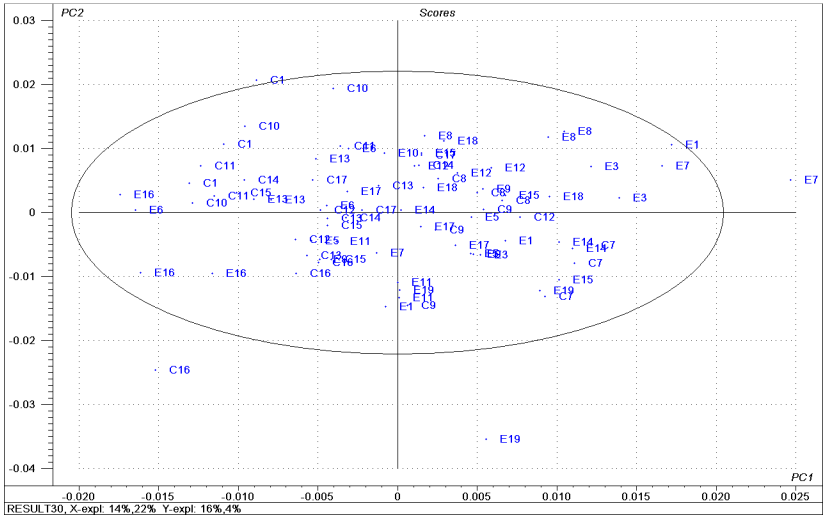
molybdenum



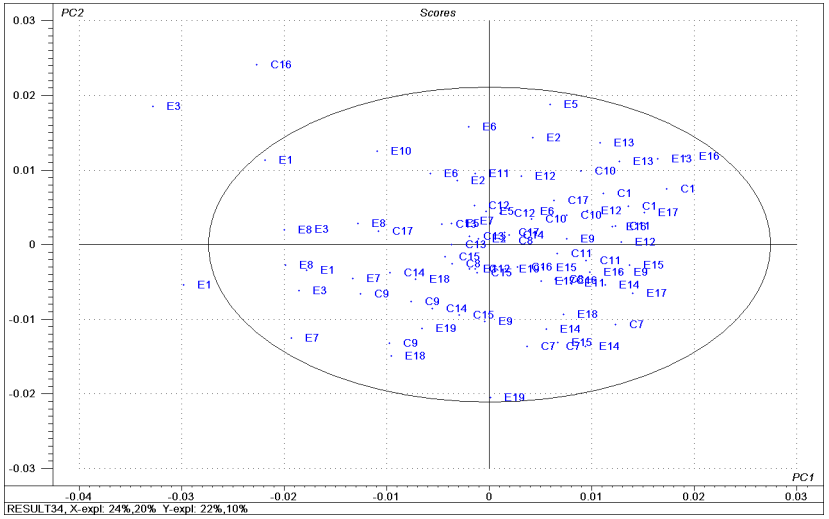
nickel



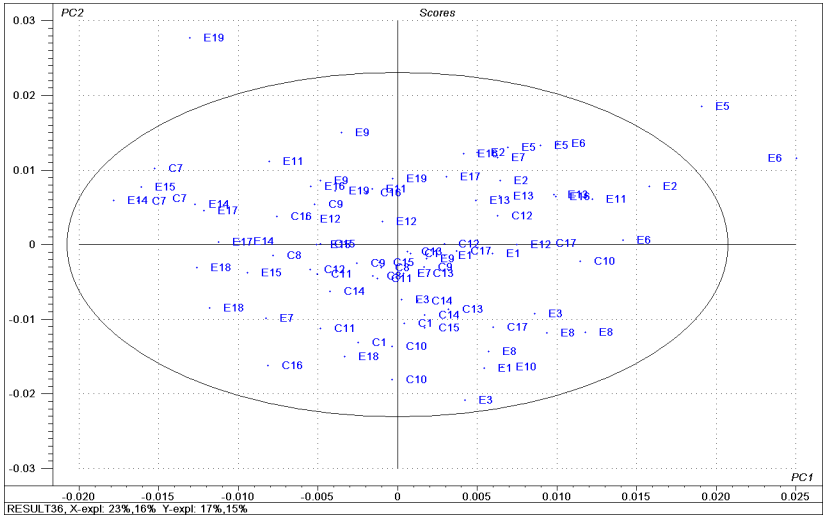
nitrate nitrogen



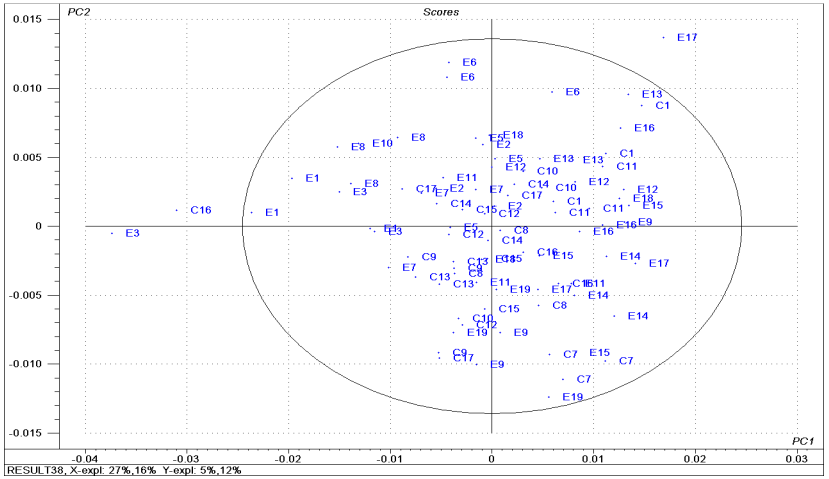
phosphorus



selenium



total iron



zinc